Time-dependent modulation of pheromone-sensitive trichoid sensilla of the hawkmoth *Manduca sexta*

Tageszeitabhängige Modulation von pheromonsensitiven Trichoidsensillen des Tabakschwärmers *Manduca sexta*

Dissertation zur Erlangung des
Doktorgrades der Naturwissenschaften
(Dr. rer. nat.)

dem Fachbereich Biologie
der Philipps-Universität Marburg
vorgelegt von
Christian Flecke
aus Schwalmstadt
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Erstgutachter: Herr Prof. Dr. Uwe Homberg
Zweitgutachterin: Frau Prof. Dr. Monika Stengl

Tag der mündlichen Prüfung am 2009.

Diese Doktorarbeit widme ich allen,
die den Unterschied zwischen
Wissenschaft und Leidenschaft kennen.

For those who know the difference
between science and passion.

Contents

Erklärung: Eigene Beiträge und veröffentlichte Teile der Arbeit

Laut §8, Absatz 3 der Promotionsordnung der Philipps-Universität Marburg (Fassung vom 28.04.1993) müssen bei den Teilen der Dissertation, welche aus gemeinsamer Forschungsarbeit entstanden sind, „die individuellen Leistungen des Doktoranden deutlich abgrenzbar und bewertbar sein". Dies betrifft die Kapitel 1-3. Die Beiträge werden im Folgenden näher erläutert.

Kapitel 1: Perfusion with cGMP analogue adapts the action potential response of pheromone-sensitive sensilla trichoidea of the hawkmoth *Manduca sexta* in a daytime-dependent manner. (Die Perfusion mit einem cGMP-Analogon adaptiert die Aktionspotentialantwort von pheromonsensitiven Trichoidsensillen des Tabakschwärmers *Manduca sexta* tageszeitabhängig.)

- Durchführung von 76,5 % der Experimente (26 von 34 Ableitungen)
- Auswertung und Analyse von 88 % der Experimente (30 von 34 Ableitungen)
- Verfassen des Manuskripts in Zusammenarbeit (Korrektur) mit Frau Prof. Dr. Monika Stengl
- Dieses Kapitel wurde in der vorliegenden Form im *Journal of Experimental Biology* veröffentlicht. (**Flecke C, Dolzer J, Krannich S, Stengl M** (2006) Perfusion with cGMP analogue adapts the action potential response of pheromone-sensitive sensilla trichoidea of the hawkmoth *Manduca sexta* in a daytime-dependent manner. *J Exp Biol* **209**:3898-3912.)

Kapitel 2: Octopamine and tyramine modulate pheromone-sensitive olfactory sensilla of the hawkmoth *Manduca sexta* in a time-dependent manner. (Oktopamin und Tyramin modulieren pheromonsensitive olfaktorische Sensillen des Tabakschwärmers *Manduca sexta* tageszeitabhängig.)

- Planung und Umbau des Ableitstandes
- Durchführung aller Experimente
- Auswertung und Analyse aller Experimente
- Verfassen des Manuskripts in Zusammenarbeit (Korrektur) mit Frau Prof. Dr. Monika Stengl
- Dieses Kapitel wurde in der vorliegenden Form im *Journal of Comparative Physiology A* veröffentlicht. (**Flecke C, Stengl M** (2009) Octopamine and tyramine modulate pheromone-sensitive olfactory sensilla of the hawkmoth *Manduca sexta* in a time-dependent manner. *J Comp Physiol A* DOI 10.1007/s00359-009-0429-4)

Kapitel 3: Perfusion with cAMP analogue affects pheromone-sensitive trichoid sensilla of the hawkmoth *Manduca sexta* in a time-dependent manner. (Die Perfusion mit einem cAMP-Analogon beeinflusst pheromonsensitive Trichoidsensillen des Tabakschwärmers *Manduca sexta* tageszeitabhängig.)

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- Auswertung und Analyse aller Experimente
- Verfassen des Manuskripts in Zusammenarbeit (Korrektur) mit Frau Prof. Dr. Monika Stengl
- Dieses Kapitel wurde in der vorliegenden Form am 24.04.2009 beim *Journal of Experimental Biology* eingereicht.

Die Abfassung der Dissertation in englischer Sprache wurde vom Dekan des Fachbereichs Biologie am 13.08.2008 genehmigt.

Zusammenfassung:

Die Fähigkeit chemische und damit auch olfaktorische Signale zu detektieren ist eine Grundvoraussetzung in der Orientierung der meisten Tierarten. Besondere Bedeutung für die Kommunikation zwischen Individuen einer Spezies spielen hierbei Pheromone, die z. B. dem Anlocken des Geschlechtspartners dienen. Gut untersucht sind die Pheromonwahrnehmung und auch die Pheromonproduktion in nacht- und tagaktiven Lepidopteren. Weibchen von *Manduca sexta* (Johannson) (Lepidoptera: Sphingidae) geben während ihrer Aktivitätsphase in der Nacht gepulste Pheromonsignale ab, die von den Männchen mit pheromonsensitiven Trichoidsensillen des Typs I detektiert werden. Diese hoch spezialisierten Sensillen befinden sich in großer Anzahl auf dem Flagellum der Antennen (Sanes und Hildebrand 1976) und ermöglichen es den Männchen die Pheromonquelle über große Entfernungen aufzuspüren. Jedes Trichoidsensillum besteht aus zwei olfaktorischen Rezeptorneuronen (ORNs), die für verschiedene Komponenten des Pheromongemisches sensitiv sind und aus drei nicht-neuronalen akzessorischen Zellen, die unterschiedliche Aufgaben innerhalb des Sensillums besitzen (Lee und Straussfeld 1990). Wie jedes sensorische System können auch ORNs von *M. sexta* auf verschiedene Duftstärken adaptieren oder sensitisieren. Einige Studien zeigten, dass die Konzentration von zyklischem Guanosinmonophosphat (cGMP) nach langen und starken Pheromonreizen in Antennenhomogenisaten und einzelnen pheromonsensitiven ORNs ansteigt (Ziegelberger et al. 1990; Boekhoff et al. 1993; Stengl et al. 2001). Deshalb wird angenommen, dass cGMP in Adaptationsprozessen von ORNs von Insekten eine entscheidende Rolle spielt. Weiterhin gibt es zahlreiche Studien die belegen, dass das biogene Amin Oktopamin (OA) in Sensitisierungsprozessen auf Ebene einzelner Trichoidsensillen mehrerer Lepidopterenarten (Pophof 2000, 2002) und in der Erhöhung der Sensitivität für Pheromon auf der Verhaltensebene beteiligt ist (Linn Jr and Roelofs 1986; Linn Jr et al. 1992). Die genauen molekularen Grundlagen dieser Modulationsmechanismen sind jedoch noch ungeklärt. Das Verhalten vieler Lepidopterenarten weist zudem tageszeitliche Rhythmen auf, die durch circadiane Schrittmacher kontrolliert werden. Dies betrifft die Pheromonproduktion, die Abgabe der Pheromone und auch die Sensitivität der Männchen für das Pheromon (Rosén 2002; Rosén et al. 2003). Für *M. sexta* wurde gezeigt, das die Pheromonabgabe der Weibchen im letzten Drittel der Nacht ihr Maximum erreicht (Itagaki und Conner 1988) und somit mit der maximalen Flugaktivität der Männchen korreliert (Sasaki und Riddiford 1984). Gesteuert werden die Rhythmen in der Pheromonsensitivität mit großer Wahrscheinlichkeit über tageszeitliche Veränderungen der OA-Konzentration in der Hämolymphe, da gezeigt wurde, dass Anstiege in der OA-Konzentration mit einer Erhöhung der Pheromonsensitivität korrelieren (Linn Jr et al. 1996). Da die meisten OA-Rezeptoren positiv an Adenylylzyklasen gekoppelt sind (Farooqui 2007), ist weiterhin anzunehmen, dass zumindest ein Teil der OA-Effekte über Anstiege der Konzentration von zyklischem Adenosinmonophosphat (cAMP) vermittelt werden. In dieser Arbeit sollte in erster Linie geklärt werden, ob auch in der Peripherie, auf Ebene einzelner ORNs tageszeitliche Unterschiede in der Sensitivität für Pheromone auftreten. Weiterhin sollte der Einfluss von zyklischen Nukleotiden und biogenen Aminen auf die Pheromontransduktion und deren Beteiligung an der tageszeitabhängigen Modulation der Pheromonsensitivität untersucht werden. Hierfür wurden extrazelluläre Langzeitableitungen von einzelnen Trichoidsensillen, sogenannte *Tip Recordings*, durchgeführt. Dabei wird eine mit einer Elektrolytlösung gefüllte Glaskapillare über ein einzelnes, zuvor gestutztes, Sensillenhaar gestülpt und somit die elektrische Aktivität des Trichoidsensillums abgeleitet. Die Sensillen wurden während der dreistündigen Ableitungen in Intervallen von 5 Minuten mit der Hauptkomponente des Pheromongemisches Bombykal (BAL) über ein Druckluftsystem stimuliert. Die Antworten auf BAL-Stimulation und die spontane Aktivität der ORNs wurden digitalisiert und später ausgewertet. Um tageszeitliche Unterschiede in der Pheromonsensitivität und in den Effekten der biogenen Amine untersuchen zu können, wurden die Ableitungen zu drei unterschiedlichen Zeitgeberzeiten (ZTs) durchgeführt, wobei ein Zeitfenster das Ende der Dunkelphase und die beiden anderen jeweils den Beginn und die Mitte des Tages abdeckten. OA, dessen Vorläufersubstanz Tyramin (TA), cGMP und cAMP wurden durch Perfusion der Sensillenlymphe über die Ableitelektrode appliziert und dessen Effekte ausgewertet. Die Arbeit gliedert sich in drei Kapitel:

Kapitel 1: **Die Perfusion mit einem cGMP-Analogon adaptiert die Aktionspotentialantwort von pheromonsensitiven Trichoidsensillen des Tabakschwärmers *Manduca sexta* tageszeitabhängig.** (Perfusion with cGMP analogue adapts the action potential response of pheromone-sensitive sensilla trichoidea of the hawkmoth *Manduca sexta* in a daytime-dependent manner.)

Es wird angenommen, dass cGMP in Adaptationsmechanismen von ORNs von Vertebraten und Invertebraten involviert ist. So wurde gezeigt, dass nach langen adaptierenden Reizen die Konzentration von cGMP in Antennenhomogenisaten von *Antheraea polyphemus* und *Bombyx mori* ansteigt, ebenso wie in einzelnen ORNs von *Manduca sexta* (Ziegelberger et al. 1990; Boekhoff et al. 1993; Stengl et al. 2001). Weiterhin reduzierte die Perfusion von isolierten Antennen von *B. mori* mit einem cGMP-Analogon das Sensillenpotential (SP) und die Aktionspotentialfrequenz in Antworten auf Pheromonstimulation (Redkozubov 2000). Dieser Effekt entspricht einem Typ der Langzeitadaptation, welcher in Ableitungen von einzelnen pheromonsensitiven Trichoidsensillen von *M. sexta* beschrieben wurde (Dolzer et al. 2003). Zudem weist die Pheromonwahrnehmung vieler männlicher Lepidopteren tageszeitabhängige Schwankungen in der Sensitivität für das von den Weibchen abgegebene Pheromon auf. Für die nachtaktiven *M. sexta* wurde gezeigt, dass die Weibchen das Pheromon hauptsächlich im letzten Drittel der Dunkelphase abgeben und zudem die Maxima in der männlichen Flugaktivität mit der Phase der Pheromonabgabe korrelieren (Sasaki und Riddiford 1984; Itagaki und Conner 1988). Um auf der einen Seite den Einfluss von cGMP auf die Pheromontransduktion von Trichoidsensillen von *M. sexta* zu untersuchen und weiterhin tageszeitliche Unterschiede in den cGMP-Effekten zu analysieren, wurden Langzeitableitungen von einzelnen Trichoidsensillen zu zwei unterschiedlichen ZTs durchgeführt. ZT 1-4 ist der Beginn der Hellphase, in dem die Tiere langsam von der aktiven in die inaktive Phase übergehen. ZT 8-11 befindet sich etwa in der Mitte des subjektiven Tages, wenn die Tiere hauptsächlich inaktiv sind. Die pheromonsensitiven Trichoidsensillen wurden während der dreistündigen Ableitungen in Intervallen von 5 Minuten mit der Hauptkomponente des Pheromongemisches BAL stimuliert (Dosis 10 µg BAL, Stimulusdauer 50 ms). Diese Art der Stimulation ist nicht adaptierend.

Die Perfusion der Sensillenlymphe mit 10 $mmol^{-1}$ des membrangängigen cGMP-Analogons 8-bromo cGMP (8bcGMP) verringerte die Anfangsaktionspotentialfrequenz in Antworten auf BAL-Stimulation signifikant in Ableitungen bei ZT 1-4 und ZT 8-11, wobei die 8bcGMP-abhängige Abnahme bei ZT 8-11 um 12 % stärker war als bei ZT 1-4. Die Perfusion mit 8bcGMP hatte jedoch keinen Einfluss auf die Generierung des SPs, wie der stabile Zeitverlauf der SP-Amplitude in Ableitungen während beider ZTs beweist. Weiterhin wurde durch den Einfluss von 8bcGMP die Amplitudenreduktion von Aktionspotentialen in Pheromonantworten abgeschwächt, dies jedoch ausgeprägt nur in Ableitungen bei ZT 8-11. Ein weiteres wichtiges Ergebnis dieser Arbeit stellen die Effekte von 8bcGMP auf die Verteilung von Aktionspotentialen in den Pheromonantworten dar. Während in den Kontrollen bei ZT 1-4 eine leichte Verschiebung hin zu mehr tonischen Antworten und eine leichte, aber signifikante Abnahme in der Anzahl der Aktionspotentiale in den ersten 100 ms der Antworten festgestellt werden konnte, war dieser Effekt für Ableitungen bei ZT 8-11 wesentlich stärker ausgeprägt. Der schwache Effekt bei ZT 1-4 und die starke Verschiebung von phasischen zu mehr tonischen Aktionspotentialantworten und die mehrfach stärkere Abnahme in der Anzahl der Aktionspotentiale in den ersten 100 ms bei ZT 8-11 deuten darauf hin, das dieser Effekt einen weiteren endogenen Adaptationsmechanismus darstellen könnte. Zwar wurde unter dem Einfluss von 8bcGMP eine Verschiebung in der Aktionspotentialverteilung hin zu mehr tonischen Antworten und eine signifikante Abnahme in den Aktionspotentialen in den ersten 100 ms nur bei ZT 8-11 gefunden, doch war der 8bcGMP-abhängige Effekt wesentlich stärker als in der assoziierten Kontrolle. Weiterhin veränderte 8bcGMP die Form spontaner Aktionspotentiale von nicht stimulierten ORNs. Nach Injektion von 8bcGMP in die Hämolymphe während Langzeitableitungen von einzelnen Trichoidsensillen vergrößerte sich die maximale Aktionspotentialamplitude beider Aktionspotential-klassen und die Repolarisationsphase der Aktionspotentiale wurde verlängert.

Unsere Ergebnisse zeigen deutlich, dass cGMP auch in ORNs von *M. sexta* an der Langzeitadaptation der Pheromontransduktion beteiligt ist. 8bcGMP hatte jedoch nur einen Einfluss auf die Aktionspotentialantwort, was unsere Ergebnisse von anderen Untersuchungen unterscheidet. Die Abnahme in der Aktionspotentialfrequenz und die Verlängerung der Repolarisationsphase spontaner

Aktionspotentiale kann durch das Öffnen von zyklisch-nukleotid-abhängigen K^+-Kanälen erklärt werden. Auch die Abschwächung der Amplitudenreduktion in den BAL-Antworten und der Anstieg der maximalen Aktionspotentialamplitude spontaner Aktionspotentiale kann durch ein Schließen von cGMP-sensitiven K^+-Kanälen und der damit einhergehenden Zunahme des Präparatewiderstandes erklärt werden. Wir nehmen an, dass starke oder lange Pheromonstimuli zu einem Anstieg der intrazellulären Ca^{2+}-Konzentration führen, welche wiederum einen Anstieg der cGMP-Konzentration zur Folge hat. Die Erhöhung des cGMP-Niveaus führt dann zum Schließen der schnellen BAL- und cGMP-abhängigen K^+-Kanäle, welche von früheren Patch-Clamp-Ableitungen von kultivierten ORNs bekannt sind (Zufall et al. 1991; Dolzer 2002). Besonders bemerkenswert an den Resultaten dieser Arbeit sind die tageszeitabhängigen Effekte von 8bcGMP. Egal, ob auf die Abnahme der Aktionspotentialfrequenz, der Abnahme der Amplitudenreduktion oder auf die Abnahme der Aktionspotentiale in den ersten 100 ms der Antworten, 8bcGMP hatte einen wesentlich stärkeren Effekt bei ZT 8-11 als bei ZT 1-4. Außerdem könnte die endogene Verschiebung der Aktionspotentialverteilung in den Kontrollableitungen bei ZT 1-4 und ZT 8-11 hin zu mehr tonischen Antworten auf einen weiteren Adaptationsmechanismus hindeuten, der durch die Applikation von exogenem cGMP zudem noch verstärkt wurde. Es wurde gezeigt, das ORNs mit tonischen Antworten nicht in der Lage sind gepulste Pheromonsignale mit höheren Frequenzen aufzulösen (Kodadová 1996), wie es für ein erfolgreiches Auffinden der Pheromonquelle notwendig ist. Ob der Adaptation auf Ebene der Aktionspotentialverteilung in den Antworten ein endogener Anstieg der cGMP-Konzentration während der Photophase zugrunde liegt, muss noch untersucht werden.

Kapitel 2: **Oktopamin und Tyramin modulieren pheromonsensitive olfaktorische Sensillen des Tabakschwärmers *Manduca sexta* tageszeitabhängig.** (Octopamine and tyramine modulate pheromone-sensitive olfactory sensilla of the hawkmoth *Manduca sexta* in a time-dependent manner.)

In zahlreichen Studien wurde gezeigt, dass das biogene Amin OA, welches in Insekten als Neuromodulator, Neurohormon und Neuromediator fungiert, die Pheromonsensitivität männlicher Lepidopteren in Verhaltensversuchen erhöht (Linn Jr and Roelofs 1986, 1992; Linn Jr et al. 1992). Zudem wurde für *M. sexta* und auch für *Trichoplusia ni* gezeigt, dass die OA-Konzentration in der Hämolymphe über den Tagesverlauf Schwankungen aufweist, welche durch circadiane Schrittmacher kontrolliert werden (Lehmann 1990; Linn Jr et al. 1994, 1996). Interessanterweise korrelieren dabei hohe OA-Konzentrationen in der Hämolymphe mit einer erhöhten Bewegungsaktivität, aber auch mit einer erhöhten Sensitivität für Pheromon. In *M. sexta* wurden während der Dunkelphase, in der das Paarungsverhalten und die Pheromonabgabe stattfinden, hohe Konzentrationen von OA in der Hämolymphe gemessen. Während in der Photophase, in der der Großteil der Tiere inaktiv ist, wesentlich geringere OA-Konzentrationen festgestellt wurden (Lehman 1990). Weiterhin wurde gezeigt, das OA eine tageszeitabhängig unterschiedliche Wirkung besitzt, führte es in Verhaltensversuchen nur dann zu einer Zunahme in der Pheromonsensitivität, wenn es während der Hellphase, aber nicht während der Dunkelphase injiziert wurde (Linn Jr and Roelofs 1986, 1992; Linn Jr et al. 1992). Um tageszeitabhängige Unterschiede in der Pheromonsensitivität der ORNs und tageszeitabhängige Effekte von OA und seiner Vorläufersubstanz TA auf die Pheromontransduktion zu untersuchen, wurden Langzeitableitungen von einzelnen Trichoidsensillen von *M. sexta* zu drei unterschiedlichen ZTs (ZT 22-1, ZT 1-4 und ZT 8-11; ZT 0 = Licht an) durchgeführt. ZT 22-1 umfasst die beiden letzten Stunden der Dunkelphase und die erste Stunde der Hellphase, während ZT 1-4 und ZT 8-11 den Beginn bzw. die Mitte der Hellphase abdecken. Um Phasenverschiebungen der circadianen Schrittmacher zu verhindern, wurden die komplette Präparation und die Ableitung in der Dunkelphase unter Rotlichtbedingungen durchgeführt. OA und TA wurden mit einer Konzentration von 10 mmol l^{-1} durch Perfusion über die Ableitelektrode appliziert. Um endogene OA-Effekte zu blockieren, wurde zudem in Versuchen bei ZT 22-1 und ZT 8-11 der OA-Rezeptor-Antagonist Epinastin (EPI) eingesetzt. Die Sensillen wurden während des Ableitzeitraums von 180 Minuten mit der Hauptkomponente des Pheromongemisches BAL (Dosis 10 oder 1 µg, Stimulusdauer 50 ms) in Intervallen von 5 Minuten stimuliert.

Die Perfusion mit OA führte zu einer Zunahme der Sensillenpotentialamplitude (SP-Amplitude) in Ableitungen bei ZT 1-4 und ZT 8-11, wobei der OA-abhängige Anstieg bei ZT 8-11 um das 9.74-fache stärker war. Auch TA erhöhte die SP-Amplitude, jedoch nur in Ableitungen bei ZT 8-11 und zudem war der OA-abhängige Anstieg um das 2.5-fache stärker. Weiterhin erhöhten OA und TA die normalisierte Aktionspotentialfrequenz jedoch nur in Ableitungen bei ZT 8-11. Um die absoluten Effekte der biogenen Amine bestimmen zu können, wurde die mittlere Aktionspotentialfrequenz für die drei betroffenen ZTs ermittelt. In den Kontrollen mit 10 und 1 µg BAL Stimulation wurde bei ZT 8-11 eine im Vergleich zu ZT 22-1 signifikant niedrigere mittlere Aktionspotentialfrequenz gemessen, was auf eine endogene Adaptation der ORNs in der Photophase hinweist. Die Perfusion mit OA und TA wirkte der Abnahme bei ZT 8-11 entgegen. Weiterhin wurden Effekte auf die Verteilung der Aktionspotentiale in den BAL-Antworten gefunden. Während in den Kontrollen bei ZT 8-11 eine deutliche Verschiebung von phasischen hin zu tonischen Antworten und eine signifikante Abnahme der Aktionspotentiale in den ersten 100 ms gefunden wurde, führte die Applikation von OA und TA zur Aufhebung dieses Adaptationsprozesses während der Photophase. Außerdem verstärkte OA, jedoch nicht TA, die Amplitudenreduktion in Ableitungen bei ZT 8-11. Weiterhin erhöhte OA, aber nicht TA, die normalisierte spontane Aktionspotentialfrequenz bei ZT 8-11 durch geringfügige Verlängerung der Bursts und durch Anstiege in der Anzahl der Bursts, wobei sich der Prozentsatz von Aktionspotentialen die als Teil eines Bursts auftraten erhöhte. Die Perfusion mit EPI reduzierte die normalisierte Aktionspotentialfrequenz in Ableitungen bei ZT 8-11 innerhalb einer Stunde beinahe vollständig, hatte jedoch keinen signifikanten Effekt auf die SP-Amplitude. Bei ZT 22-1 führte die Applikation von EPI mit einer 10-fach höheren Konzentration als bei ZT 8-11 nur zu einer schwachen Abnahme der Aktionspotentialfrequenz. EPI verringerte zudem die spontane Aktionspotentialfrequenz in Ableitungen bei ZT 22-1 und bei ZT 8-11, wobei beide Effekte in ihrer Stärke relativ ähnlich waren. Zusätzlich führte die Applikation von EPI bei ZT 22-1 zu einer Verschiebung von phasischen hin zu tonischen Aktionspotentialantworten und zu einer signifikanten Abnahme der Aktionspotentiale in den ersten 100 ms der BAL-Antworten. Dieser EPI-abhängige Effekt trat auch in Ableitungen bei ZT 8-11 auf, war jedoch wesentlich stärker ausgeprägt.

In dieser Studie zeigten wir zum ersten Mal, das pheromonsensitive ORNs von *Manduca sexta* während der Photophase auf Ebene der Aktionspotentialfrequenz adaptieren und das die Perfusion mit OA und TA zur Disadaptation der ORNs während der Photophase führt. Nach Applikation von OA öffnen vermutlich vermehrt Kanäle im Dendriten, was den Anstieg der SP-Amplitude und der Aktionspotentialfrequenz bedingt. Hierdurch lässt sich auch die Zunahme der Amplitudenreduktion der Aktionspotentiale in den BAL-Antworten erklären, da einhergehend mit dem vermehrten Öffnen von Ionenkanälen auch der Präparatewiderstand abnimmt. Weiterhin ist davon auszugehen, dass der Rezeptor über den der OA-Effekt vermittelt wird, eher ein OA- als ein TA-Rezeptor ist. Zum einen ist die größte Zahl von OA-Rezeptoren im Gegensatz zu TA-Rezeptoren positiv an Adenylylzyklasen gekoppelt (Farooqui 2007) und zweitens war der Effekt von OA weitaus stärker als der TA-Effekt. Zudem wurde in Antennen von *M. sexta* ein alpha-adrenerg-ähnlicher OA-Rezeptor nachgewiesen (Dacks et al. 2006). Für diesen Typ Rezeptor wurde gezeigt, dass er die intrazelluläre Konzentrationen von cAMP und Ca^{2+} erhöht und außerdem eine höhere Affinität für OA als für TA besitzt. Die fehlenden Effekte von EPI auf die SP-Amplitude und die Unterschiede in den OA- und TA-Effekten weisen außerdem darauf hin, das mehrere unterschiedliche OA oder OA/TA-Rezeptoren an der Steuerung der Pheromonsensitivität beteiligt sein können. Besonders merkenswert in dieser Studie waren wiederum die tageszeitabhängigen Effekte. Die Perfusion mit OA zeigte nur bei ZT 8-11 eine starke Wirkung, war aber in der Dunkelphase bei ZT 22-1 wirkungslos. Demnach ist die Applikation von OA nur dann effektiv, wenn in der Hämolymphe eine geringe OA-Konzentration vorliegt, wie es bei ZT 8-11 der Fall ist. Interessanterweise korrelieren die gezeigten Veränderungen in der OA-Hämolymphkonzentration mit der Stärke der OA-Effekte, aber auch mit der Sensitivität der ORNs, konnten wir doch zeigen das die mittlere Aktionspotentialfreuqenz in den Antworten bei ZT 8-11 wesentlich geringer ist als bei ZT 22-1. Das Fehlen eines Effektes während der Dunkelphase könnte dadurch bedingt sein, dass die OA-Rezeptoren während hoher OA-Konzentrationen adaptieren oder dass ein anderer Mechanismus, wie die tageszeitlich bedingte Veränderung in der Anzahl von Pheromonrezeptoren, dem OA-Effekt entgegenwirkt. Weiterhin beeinflusste OA die Fähigkeit von ORNs Pheromonpulse zeitlich aufzulösen. Es ist bekannt, dass ORNs die Pheromonantworten mit einer

tonischen Aktionspotentialverteilung generieren wesentlich geringere Frequenzen des gepulsten Pheromonsignals auflösen können (Almaas et al. 1991; Kodadová 1996). Erstens wirkten OA und TA der endogenen Verschiebung hin zu mehr tonischen Aktionspotentialantworten bei ZT 8-11 entgegen und zweitens wurden in Ableitungen bei ZT 22-1, wenn hohe endogene OA-Konzentrationen vorlagen, durchweg phasische Aktionspotentialantworten gemessen. Wir nehmen an, dass ein gewisses Niveau von OA in der Hämolymphe vorliegen muss, damit starke Reizanworten mit einer phasischen Aktionspotentialverteilung generiert werden können. Diese Annahme wird außerdem dadurch bestätigt, dass die Applikation von EPI bei ZT 8-11 aber auch bei ZT 22-1 zu einer Verschiebung zu mehr tonischen Antworten geführt hat und zudem die Aktionspotentialfrequenz bei ZT 8-11 stark und bei ZT 22-1 leicht herabgesetzt wurde. Wir zeigten außerdem, dass OA die spontane Aktionspotentialaktivität durch Verlängerung der Bursts und durch Verringerung der Interburst-Intervalle erhöht. Unsere Ergebnisse stützen damit andere Untersuchungen (Olianas et al. 2005), die belegen, dass die OA-abhängigen Effekte auf das spontane Burstverhalten ein generelles Charakteristikum von OA in verschiedenen Geweben darstellt. Da die spontane Aktionspotentialaktivität in dieser Untersuchung zwischen den Reizen gemessen wurde und zudem in den Kontrollen eine leichte Abnahme der spontanen Aktionspotentialfrequenz auftrat, kann von einer temporären Adaptation der spontanen Aktionspotentialaktivität bei ZT 8-11 ausgegangen werden. Da OA diese Adaptation aufhebt und die spontane Aktivität bei ZT 8-11 erhöht, bestätigt dies unsere Hypothese, dass OA ORNs disadaptiert und nicht sensitisiert. Schlussfolgernd stellten wir die Hypothese auf, das OA obligatorisch für die Detektion von gepulsten Pheromonsignalen zu jeder ZT ist.

Kapitel 3: **Die Perfusion mit einem cAMP-Analogon beeinflusst pheromonsensitive Trichoidsensillen des Tabakschwärmers *Manduca sexta* tageszeitabhängig.** (Perfusion with cAMP analogue affects pheromone-sensitive trichoid sensilla of the hawkmoth *Manduca sexta* in a time-dependent manner.)

Es wurde gezeigt, dass der second messenger cAMP in Vertebraten sowie in Invertebraten an olfaktorischen Prozessen beteiligt ist. Während die Rolle von cAMP in der Transduktion von ORNs von Vertebraten gut untersucht ist, ist die Bedeutung von cAMP für die Transduktions- und Modulationsmechanismen von ORNs von Insekten weitestgehend ungeklärt. So wurde z. B. nach Perfusion mit cAMP in Antennen von *A. polyphemus* eine Erhöhung der Amplituden von Elektroantennogrammen (EAGs) auf Pheromonstimulation gemessen (Villet 1978). In *Drosophila melanogaster* führte die Überexpression des *dunce*-Gens, welche eine Phosphodiesterase kodiert, zu Veränderungen in EAGs (Martín et al. 2001) und zu einer Abnahme der Empfindlichkeit für Ethanol in Verhaltensversuchen (Gomez-Diaz et al. 2004). Weiterhin wurde gezeigt, dass cAMP an einer Verstärkung von schwachen Duftsignalen über eine cAMP-abhängige Aktivierung des ubiquitären olfaktorischen Rezeptors (OR) OR83b beteiligt ist (Wicher et al. 2008). Zudem zeigten wir in Kapitel 2, dass OA pheromonsensitive ORNs von *M. sexta* tageszeitabhängig disadaptiert (Farooqui 2007, Flecke and Stengl 2009). Da die meisten OA-Rezeptoren unter anderem positiv an Adenylylzyklasen gekoppelt sind, ist die Wahrscheinlichkeit sehr hoch, das zumindest ein Teil der OA-abhängigen Effekte über Anstiege in der cAMP-Konzentration vermittelt wird. Um zu untersuchen, ob cAMP an der Transduktion von pheromonsensitiven Trichoidsensillen beteiligt ist und um die Rolle von cAMP in der tageszeitabhängigen Modulation der Sensitivität von ORNs von *M. sexta* zu klären, wurden extrazelluläre Langzeitableitungen von einzelnen Trichoidsensillen zu drei unterschiedlichen ZTs durchgeführt (ZT 22-1, ZT 1-4 und ZT 8-11). Die Ableitungen innerhalb der Dunkelphase wurden dabei unter Rotlichtbedingungen durchgeführt. Das membrangängige cAMP-Analogon 8-bromo cAMP (8bcAMP) wurde in einer Konzentration von 10 mmol l^{-1} durch Perfusion über die Ableitelektrode mit Beginn der Ableitungen appliziert. Die Trichoidsensillen wurden während der Ableitdauer von 180 Minuten mit der Hauptkomponente des Pheromongemisches BAL in Intervallen von 5 Minuten stimuliert (Dosis 10 oder 1 µg BAL, Stimulusdauer 50 ms).

Die Perfusion mit 8bcAMP erhöhte die normalisierte SP-Amplitude in Ableitungen mit 10 und 1 µg BAL Stimulation bei ZT 1-4 und ZT 8-11 aber nicht bei ZT 22-1. Im Gegensatz dazu hatte 8bcAMP keinen Effekt auf die normalisierte Aktionspotentialfrequenz. Um die absoluten 8bcAMP-Effekte bestimmen zu können, wurde die mittlere SP-Amplitude und die mittlere Aktionspotentialfrequenz über die absoluten Werte für jede ZT berechnet. In den Kontrollen mit 10 µg BAL Stimulation wurde dabei während der Photophase bei ZT 1-4 und ZT 8-11 eine wesentlich niedrigere SP-Amplitude gemessen als bei ZT 22-1. Die Applikation von 8bcAMP in Ableitungen mit 10 µg BAL Stimuli erhöhte die mittlere SP-Amplitude bei ZT 1-4 und ZT 8-11, während in Ableitungen mit 1 µg BAL nur bei ZT 1-4 eine Zunahme der mittleren SP-Amplitude, bei ZT 8-11 jedoch eine Abnahme ermittelt wurde. Auch auf Ebene der mittleren Aktionspotentialfrequenz wurde, wie früher gezeigt, in den Kontrollen bei ZT 8-11 eine im Vergleich zu ZT 22-1 wesentlich niedrigere Aktionspotentialfrequenz gemessen. Die Perfusion mit 8bcAMP führte bei ZT 1-4 in Ableitungen mit beiden BAL-Dosen zu einer signifikanten Zunahme der mittleren AP-Frequenz, konnte jedoch die endogene Abnahme bei ZT 8-11 nicht kompensieren. Auch auf die Aktionspotentialverteilung in den Antworten hatte 8bcAMP nur marginale Effekte. Weder auf die signifikanten Abnahmen in der Anzahl der Aktionspotentiale in den ersten 100 ms der Antworten bei ZT 1-4 und ZT 8-11 noch auf die starke endogene Verschiebung von phasischen zu tonischen BAL-Antworten bei ZT 8-11 hatte 8bcAMP einen Einfluss. Weiterhin erhöhte 8bcAMP die normalisierte spontane Aktionspotentialfrequenz, jedoch nur bei ZT 8-11 und nur in Ableitungen mit 1 µg BAL Stimulation. Um die absoluten Effekte auf die spontane Aktionspotentialaktivität zu untersuchen, wurde die mittlere spontane Aktionspotentialfrequenz über die absoluten Werte für die drei unterschiedlichen ZTs ermittelt. In den Kontrollen mit 10 und 1 µg BAL kam es zu signifikanten Abnahmen in der mittleren spontanen Aktionspotentialfrequenz bei ZT 1-4 und ZT 8-11. Die Perfusion mit 8bcAMP wirkte diesem endogenen Effekt entgegen, wurden zwischen den drei ZTs doch keine signifikanten Unterschiede in Ableitungen mit beiden BAL-Dosen gefunden. Die Applikation von 1 mmol l^{-1} OA führte bei ZT 1-4 auch zu einer Kompensation der Abnahme, erhöhte jedoch bei ZT 8-11 die mittlere spontane Aktionspotentialfrequenz. Auch in der mittleren Anzahl der Bursts pro bin und der Aktionspotentiale pro bin kam es in den Kontrollableitungen mit 10 und 1 µg BAL zu signifikanten Abnahmen bei ZT 8-11 und für Ableitungen mit 1 µg BAL auch bei ZT 1-4. Die Perfusion mit 8bcAMP kompensierte diese Verschiebung zu niedrigeren Werten in Ableitungen mit 10 und 1 µg BAL Stimulation. Auch die Applikation von OA führte bei ZT 1-4 ebenfalls zu einer Aufhebung der Adaptation, erhöhte jedoch bei ZT 8-11 die mittlere Anzahl der Bursts pro bin und der Aktionspotentiale pro bin, wobei die Zunahme der Bursts um das 1.4-fache stärker war.

Mit dieser Arbeit zeigten wir zum ersten Mal dass cAMP an der tageszeitlich gesteuerten Modulation der Pheromontransduktion auf Ebene einzelner ORNs beteiligt ist. Unsere Ergebnisse bestätigen die Annahme, dass die OA-Effekte zumindest zum Teil über die Aktivierung einer Adenylylzyklase vermittelt werden. 8bcAMP könnte demnach entweder direkt über eine Interaktion mit der cAMP-sensitiven Bindestelle am OR83b wirken (Wicher et al. 2008) oder aber indirekt über eine Protein-Kinase A Ionenkanäle phosphorylieren, die an der Generierung des SPs beteiligt sind. Weiterhin ist ein indirekter Effekt über die in den akzessorischen Zellen befindlichen V-ATPasen möglich (Klein und Zimmermann 1991; Klein 1992). So könnte deren cAMP-abhängige Aktivierung zu einem Anstieg der K^+-Konzentration in der Sensillenlymphe führen, welche die *Driving Force* für das SP und damit dessen maximale Amplitude erhöht. Auf der anderen Seite gibt es jedoch auch starke Unterschiede in der Wirkung von 8bcAMP und OA. Im Gegensatz zu OA hatte 8bcAMP keinen Einfluss auf die normalisierte Aktionspotentialfrequenz und kompensierte nicht die endogene Abnahme der mittleren Aktionspotentialfrequenz bei ZT 8-11. Auch auf die Verteilung von Aktionspotentialen in den BAL-Antworten und deren Verschiebung hin zu mehr tonischen Antworten bei ZT 8-11 hatte 8bcAMP keine Wirkung. Besonders bemerkenswert ist, dass der Anstieg in der normalisierten SP-Amplitude nicht in einen Anstieg der normalisierten Aktionspotentialfrequenz umgesetzt wurde. Wir nehmen an, das der 8bcAMP-abhängige Anstieg der SP-Amplitude entweder nicht am Aktionspotentialgenerator ankommt oder aber ein zusätzlicher Adaptationsmechanismus (Dolzer et al. 2003) dem Anstieg der Aktionspotentialfrequenz entgegenwirkt. So könnte z. B. die Aktivierung eines hyperpolarisations- und zyklisch-nukleotid-gesteuerten I_h Stroms zu einer lokalen Abnahme des Eingangswiderstandes (Dibattista

et al. 2008) führen, was zu einer Abschwächung des Rezeptorpotentials während dessen Weiterleitung führen könnte. Weiterhin ist denkbar, das OA einen zweiten zusätzlichen Signalweg aktiviert, in dem eine Erhöhung der intrazellulären Ca^{2+}-Konzentration die Generierung von Aktionspotentialen verstärkt. Wobei es jedoch wahrscheinlich ist, das diese Art der Sensitisierung nur an einer langsamen Modulation, aber nicht an schnellen Pheromondosis-abhängigen Veränderungen beteiligt ist. Ein weiterer Unterschied in der Wirkung von OA und 8bcAMP zeigte sich in deren Effekt auf die spontane Aktionspotentialaktivitiät. Während OA vor allem das Burstverhalten der ORNs über eine Zunahme der Bursts und über die Verlängerung der Bursts beeinflusste, hatte die Perfusion mit 8bcAMP eine gleichstarke Zunahme in der Anzahl der Bursts und der einzeln auftretenden Aktionspotentiale zur Folge. Der 8bcAMP-abhängige Effekt kann durch die Aktivierung von I_h-Kanälen und eine damit einhergehende Verschiebung des Ruhepotentials zu positiveren Werten erklärt werden (Dibattista et al. 2008). Dadurch würden mehr Depolarisationen den Schwellenwert übersteigen. Weiterhin ist eine direkte Wechselwirkung mit der cAMP-sensitiven Bindestelle des OR83b möglich (Wicher et al. 2008), wodurch die Zahl der Depolarisationen erhöht würde. Die physiologische Bedeutung der Veränderungen auf Ebene der spontanen Aktionspotentialaktivitiät ist jedoch unbekannt. Wir haben zudem gezeigt, dass neben OA auch 8bcAMP an der tageszeitlichen Disadaptation von ORNs beteiligt ist. Die Perfusion mit 8bcAMP hatte nur einen Effekt während der Photophase, wenn die ORNs adaptiert waren, jedoch nicht in der Dunkelphase wenn die ORNs sensitisiert vorlagen. Während die OA-Effekte mit Unterschieden in der OA-Hämolymphkonzentration korrelieren (Kapitel 2, Flecke and Stengl 2009), ist eine Erklärung der unterschiedlich starken 8bcAMP-Effekte durch tageszeitliche Veränderungen in der endogenen cAMP-Konzentration mangels Daten noch nicht möglich. Neben den Gemeinsamkeiten in den 8bcAMP- und OA-Effekten wurden jedoch auch starke Unterschiede in der tageszeitabhängigen Wirkung beider Substanzen festgestellt. So hat 8bcAMP bei ZT 1-4 und ZT 8-11 eine gleich starke Zunahme der SP-Amplitude zur Folge, während OA nur bei ZT 8-11 eine starke Wirkung auf die SP-Amplitude zeigte. Zudem war der 8bcAMP-abhängige Effekt auf die spontane Aktionspotentialaktivität bei ZT 8-11 wesentlich schwächer als der OA-Effekt. Deshalb ist anzunehmen, dass die Regulation in der Sensitivität für Pheromon wahrscheinlich auf Ebene der OA-Rezeptoren gesteuert wird und dass OA neben der Wirkung über cAMP noch einen zweiten zusätzlichen Mechanismus aktiviert. Wir untersuchen zurzeit, ob auch in der cAMP-Konzentration in den Antennen tageszeitliche Unterschiede auftreten und ob OA eine Adenylylzyklase in den Antennen aktiviert. Die Ergebnisse dieser Studie unterstützen wiederum die Hypothese, dass die Regulation der Sensitivität für Pheromone auch auf Ebene einzelner ORNs stattfindet. So adaptieren die ORNs auch in der mittleren SP-Amplitude während der Photophase. Weiterhin zeigten wir zum ersten Mal, das auch in der spontanen Aktionspotentialaktivität während der Photophase eine Adaptation stattfindet, welche zudem durch OA und 8bcAMP aufgehoben werden konnte.

Fazit aus Kapitel 1-3: Die Untersuchung der tageszeitlichen Modulation von pheromonsensitiven Trichoidsensillen von *M. sexta* brachte viele neue Erkenntnisse, warf aber auch neue Fragen auf, die es gilt in zukünftigen Studien zu beantworten. Zum ersten Mal konnten wir zeigen, dass tageszeitliche Verschiebungen in der Sensitivität für Pheromon auch auf Ebene einzelner ORNs stattfinden. Dies bestätigt die Annahme, dass bereits in der Peripherie des olfaktorischen Systems von *M. sexta* circadiane Schrittmacher vorhanden sind und somit auch die Sensitivität für Pheromone durch Modulation der primären Transduktionskaskade reguliert wird. Wir konnten weiterhin zeigen, dass cGMP tageszeitabhängig ORNs auf Ebene der Aktionspotentialantwort adaptiert. Ob der endogenen Adaptation der ORNs während der Photophase eine Zunahme der cGMP-Konzentration in der Antenne zugrunde liegt und welcher Mechanismus in *M. sexta* für die Adaptation des SPs zuständig ist, muss noch genauer geklärt werden. Auch zeigten wir, dass OA die ORNs während der Photophase, wenn niedrige endogene OA-Konzentrationen in der Hämolymphe vorliegen, disadaptiert und dass eine gewisse Konzentration an OA in den Trichoidsensillen vorhanden sein muss, damit starke Pheromonantworten mit einer phasischen Aktionspotentialverteilung generiert werden können. Die OA-Effekte werden dabei zum Teil über cAMP-Anstiege vermittelt, wobei es aber sehr wahrscheinlich ist, das noch ein weiterer zusätzlicher Mechanismus von OA aktiviert wird, der die ORNs auf der Ebene der Aktionspotentialantwort

disadaptiert. Dieser potentielle Signalweg muss noch genauer untersucht werden. Wir haben zudem gezeigt, das auch in der spontanen Aktionspotentialaktivität während der Photophase eine Adaptation stattfindet und das die ORNs auch in der spontanen Aktionspotentialfrequenz durch OA und in schwächerem Maße durch cAMP disadaptiert werden. Möglicherweise hat diese Veränderung des Burstsverhaltens einen Einfluss auf die Pulsfolgenauflösung der ORNs. Zudem wurden tageszeitliche Verschiebungen in der Aktionspotentialverteilung in Pheromonantworten gefunden, die darauf hindeuten, dass während der Photophase eine geringere Pulsfolgenauflösung des Pheromonsignals möglich ist. Dieser möglicherweise zusätzliche Adaptationsmechanismus wird durch OA und TA aufgehoben. Ob dieser Mechanismus tatsächlich die zeitliche Pulsfolgenauflösung der ORNs beeinflusst und welche Rolle OA in diesem Modulationsprozess spielt, muss noch näher untersucht werden.

Referenzen:

Almaas TJ, Christensen TA, Mustaparta H (1991) Chemical communication in heliothine moths: I. Antennal receptor neurons encode several features of intra- and interspecific odorants in the male corn earworm moth *Helicoverpa zea*. J Comp Physiol A 169:249-258

Boekhoff I, Seifert E, Göggerle S, Lindemann M, Krüger BW, Breer H (1993) Pheromone-induced second-messenger signaling in insect antennae. Insect Biochem Molec Biol 23:757-762

Dacks AM, Dacks JB, Christensen TA, Nighorn AJ (2006) The cloning of one putative octopamine receptor and two putative serotonin receptors from the tobacco hawkmoth *Manduca sexta.* Insect Biochem Mol Biol 36:741-747

Dibattista M, Mazzatenta A, Grassi F, Tirindelli R, Menini, A (2008) Hyperpolarization-activated cyclic nucleotide-gated channels in mouse vomeronasal sensory neurons. J Neurophysiol 100:576-586

Dolzer J (2002) Mechanisms of modulation and adaptation in pheromone-sensitive trichoid sensilla of the hawkmoth *Manduca sexta*. Ph.D. Thesis Philipps-Universität, Marburg, Germany http://archiv.ub.uni-marburg.de/diss/z2002/0185

Dolzer J, Fischer K, Stengl M (2003) Adaptation in pheromone-sensitive trichoid sensilla of the hawkmoth *Manduca sexta*. J Exp Biol 206:1575-1588

Flecke C, Stengl M (2009) Octopamine and tyramine modulate pheromone-sensitive olfactory sensilla of the hawkmoth *Manduca sexta* in a time-dependent manner. J Comp Physiol A DOI 10.1007/s00359-009-0429-4

Farooqui T (2007) Octopamine-mediated neuromodulation of insect senses. Neurochem Res 32:1511-1529

Gomez-Diaz C, Martín F, Alcorta E (2004) The cAMP transduction cascade mediates olfactory reception in *Drosophila melanogaster*. Behav Genet 34:395-406

Itagaki H, Conner WE (1988) Calling Behavior of *Manduca sexta* (L.) (Lepidoptera: Sphingidae) with Notes on the Morphology of the Female Sex Pheromone Gland. Ann Ent. Soc Am 81:798-807

Klein U (1992) The insect V-ATPase, a plasma membrane proton pump energizing secondary active transport: immunological evidence for the occurrence of a V-ATPase in insect ion-transporting epithelia. J Exp Biol 172:345-354

Klein U, Zimmermann B (1991) The vacuolar-type ATPase from insect plasma membrane: immunocytochemical localization in insect sensilla. Cell Tissue Res 266:265-273

Kodadová B (1996) Resolution of pheromone pulses in receptor cells of *Antheraea polyphemus* at different temperatures. J Comp Physiol A 179:301-310

Lee JK, Strausfeld NJ (1990) Structure, distribution, and number of surface sensilla and their receptor cells on the antennal flagellum of the male sphinx moth *Manduca sexta*. J Neurocytol 19:519-538

Lehman HK (1990) Circadian control of *Manduca sexta* flight. (Abstract) Society For Neuroscience Abstracts, Volume 16, 1334

Linn CE Jr, Roelofs WL (1986) Modulatory effects of octopamine and serotonin on male sensitivity and periodicity of response to sex pheromone in the cabbage looper moth, *Trichoplusia ni*. Arch Insect Biochem Physiol 3:161-171

Linn CE Jr, Roelofs WL (1992) Role of photoperiod cues in regulating the modulatory action of octopamine on pheromone-response thresholds in the cabbage looper moth. Arch Insect Biochem Physiol 20:285-302

Linn CE Jr, Campbell MG, Roelofs WL (1992) Photoperiod cues and the modulatory action of octopamine and 5-hydroxytryptamine on locomotor and pheromone response in male gypsy moths, *Lymantria dispar*. Arch Insect Biochem Physiol 20:265-284

Linn CE Jr, Poole KR, Roelofs WL (1994) Studies on biogenic amines and their metabolites in nervous tissue and hemolymph of adult male cabbage looper moths-I. Quantitation of photoperiod changes. Comp Biochem Physiol 108:73-85

Linn CE Jr, Campbell MG, Poole KR, Wu W-Q, Roelofs WL (1996) Effects of photoperiod on the circadian timing of pheromone response in male *Trichoplusia ni*: Relationship to the modulatory action of octopamine. J Insect Physiol 42:881-891

Martín F, Charro MJ, Alcorta E (2001) Mutations affecting the cAMP transduction pathway modify olfaction in *Drosophila*. J Comp Physiol A 187:359-370

Olianas MC, Solari P, Garau L, Liscia A, Crnjar R, Onali P (2005) Stimulation of cyclic AMP formation and nerve electrical activity by octopamine in the terminal abdominal ganglion of the female gypsy moth *Lymantria dispar*. Brain Res 1071:63-74

Pophof B (2000) Octopamine modulates the sensitivity of silkmoth pheromone receptor neurons. J Comp Physiol A 186:307-313

Pophof B (2002) Octopamine enhances moth olfactory responses to pheromones, but not those to general odorants. J Comp Physiol A 188:659-662

Redkozubov A (2000) Guanosine 3′,5′-cyclic monophosphate reduces the response of the Moth's olfactory receptor neuron to pheromone. Chem Senses 25:381-385

Rosén WQ (2002) Endogenous control of circadian rhythms of pheromone production in the turnip moth *Agrotis segetum*. Arch Insect Biochem Physiol 50:21-30

Rosén WQ, Han GB, Löfstedt C (2003) The circadian rhythm of the sex-pheromone-mediated behavioral response in the turnip moth, *Agrotis segetum*, is not controlled at the peripheral level. J Biol Rhythms 18:402-408

Sanes JR, Hildebrand JG (1976) Structure and development of antennae in a moth, *Manduca sexta*. Dev Biol 51:280-299

Sasaki M, Riddiford LM (1984) Regulation of reproductive behaviour and egg maturation in the tobacco hawk moth, *Manduca sexta.* Physiol Entomol 9:315-327

Sato K, Pellegrino M, Nakagawa T, Nakagawa T, Vosshall LB, Touhara K (2008) Insect olfactory receptors are heteromeric ligand-gated ion channels. Nature 452:1002-1006

Stengl M, Zintl R, de Vente J, Nighorn A (2001) Localization of cGMP-immunoreactivity and of soluble guanylyl cyclase in antennal sensilla of the hawkmoth *Manduca sexta*. Cell and Tissue Res 304:409-421

Villet RH (1978) Mechanism of insect sex-pheromone sensory transduction: role of adenylate cyclase. Comp Biochem Physiol 61:389-394

Wicher D, Schäfer R, Bauernfeind R, Stensmyr MC, Heller R, Heinemann SH, Hansson BS (2008) *Drosophila* odorant receptors are both ligand-gated and cyclic-nucleotide-activated cation channels. Nature 452:1007-1011

Ziegelberger G, van den Berg MJ, Kaissling KE, Klumpp S, Schultz JE (1990) Cyclic GMP levels and guanylate cyclase activity in pheromone-sensitive antennae of the silkmoths *Antheraea polyphemus* and *Bombyx mori*. J Neurosci 10:1217-1225

Zufall F, Stengl M, Franke C, Hildebrand JG, Hatt H (1991) Ionic currents of cultured olfactory receptor neurons from antennae of male *Manduca sexta*. J Neurosci 11:956-965

Introduction

The ability to detect and respond to chemical signals in an appropriate manner serves as the primary window to the sensory world for most animal species. Odours of various origins signal the animal where to find food, water and conspecifics or help them to avoid harmful environments. Most important for the intraspecific communication are pheromones, which are chemical messengers emitted and perceived by individuals of the same species, playing different roles in a variety of behavioural contexts. For example recognition pheromones which denote the identity and social status; aggregation pheromones which mediate feeding and aggression, including trail following and food-marking pheromones; and most important for this study, sex pheromones triggering courtship behaviour. The composition and identity of sex pheromones, the olfactory organs for detection and the neural networks processing olfactory information have been extensively studied in different moth species, including the hawkmoth *Manduca sexta*. The sensory system of male *M. sexta* is highly sensitive to pulsed pheromone signals emitted by females, enabling the male moths to detect pheromone sources over long distances. To avoid overstimulation and to adjust olfactory sensors to odour stimuli covering concentrations over several decades, the olfactory receptors and processing neurons can adapt or sensitize to stimuli of different strength, thereby increasing the dynamic range of the sensory system without loss of resolution. Biogenic amines and cyclic nucleotides are supposed to play an important role in the modulation of pheromone transduction. The molecular basis for these adaptation- and sensitization-mechanisms in pheromone sensitive sensilla is still not fully understood. Furthermore, many physiological processes, also in sensory systems, show time-dependent differences in its metabolic rates or in its sensitivity to stimulation. These time-dependent variations were shown to be part of circadian rhythms, controlled by central or peripheral circadian pacemakers. Olfactory rhythms in moths were found on the behavioural level and in the sensitivity to pheromone. However, it was unclear whether these rhythms and the time-dependent differences additionally originate from peripheral regulatory processes in the antennae or are the result of circadian rhythms in higher processing neuropils, like the antennal lobe or the mushroom bodies. The primary aim of this Ph.D. thesis was to search for time-dependent variations in the pheromone-sensitivity on the level of single olfactory receptor neurons (ORNs) and to elucidate the roles of biogenic amines and cyclic nucleotides in time-dependent adaptation- and sensitization-processes of the peripheral olfactory sensory system of *M. sexta*.

The peripheral olfactory system of *Manduca sexta*

The antennae are the most important odour-sensitive organs in insects. Antennae of both female and male *M. sexta* comprise three segments, two small basal segments (scape and pedicel) and a long distal flagellum (Sanes and Hildebrand 1976). In both genders the antennal flagellum, which is characterized by a strong sexual dimorphism, is about 2 cm long and is divided into 80 or more annuli (Sanes and Hildebrand 1976). Males of *M. sexta* are able to detect pheromone signals emitted by females with specialized trichoid sensilla type I. These very long sensilla (sensilla hairs about 400 µm) are located in a number of about 43.000 on each flagellum of the antennae and are arranged in U-shaped patterns on each annulus (Sanes and Hildebrand 1976). In contrast, on female antennae no pheromone sensitive trichoid sensilla were found. Next to pheromone-sensitive sensilla the antennae also contain non-pheromone odour-, mechano- and hygrosensitive sensilla and comprise contact chemoreceptors, but at a lower number than trichoid sensilla.

Structure of pheromone-sensitive trichoid sensilla

Pheromone-sensitive trichoid sensilla of moth species are composed of at least two bipolar neuronal and three non-neuronal accessory cells, the tormogen, trichogen and thecogen cell (Fig. 1) (Sanes and Hildebrand 1976; Keil 1989; Lee and Strausfeld 1990). Each trichoid sensillum of *M. sexta* houses two ORNs which are sensitive to different components of the pheromone blend. One of the ORNs responds to stimulation with the main pheromone component bombykal [BAL, (*E,Z*)-

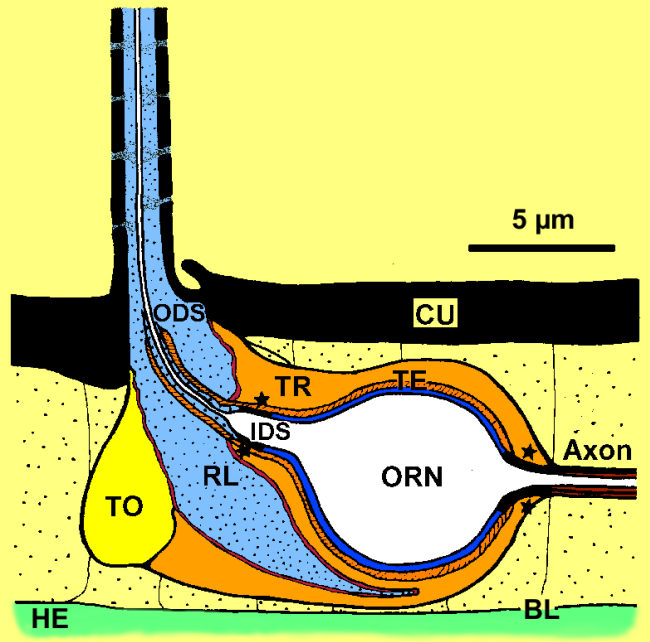

Fig. 1: Schematic diagram of a trichoid sensillum of *M. sexta*. Shown is only one of the two ORNs. Next to the ORNs each sensillum also comprises three non-neuronal cells with different functions. (ODS: outer dendritic segment; CU: cuticle; TR: trichogen cell; TE: thecogen cell; TO: tormogen cell; RL: receptor lymph cavity; IDS: inner dendritic segment; ORN: olfactory receptor neuron; HE: hemolymph; BL: basal lamina: *: septate junctions)

10,12-hexadecadienal] and the other mainly to stimulation with one of two isomeric hexadecatrienals (Kalinová et al. 2001). In extracellular tip recordings of trichoid sensilla, action potentials from both ORNs can be distinguished by their different peak-to-peak amplitudes, whereas the action potentials with larger amplitudes are always generated by the BAL-sensitive ORN (Dolzer et al. 2001). The dendrites of the ORNs are separated into an outer and an inner dendritic segment by a short ciliary structure (Keil 1989). The outer dendritic segments of both ORNs project into the very long sensillar hair, where transduction processes take place. The somata of both ORNs and the inner dendritic segments are tightly enveloped by the thecogen cell, thus isolating both ORNs from each other. In addition, the different compartments of the ORNs are separated by septate junctions between the membranes of the ORNs and the tormogen cell (Keil 1989). During development the trichogen cell together with the tormogen cell secrete the cuticle which forms the long cuticular hair shaft (Sanes and Hildebrand 1976). In addition, both cells form an inner cavity, the sensillar lymph space and also contribute to its composition (Sanes and Hildebrand 1976; Keil 1989). Especially the high K^+-concentration of the sensillar lymph, which is assumed to originate from the activity of V-ATPases located in the accessory cells (Wieczorek 1991; Wieczorek et al. 2003), is important to build up the transepithelial potential between the sensillar lymph and the hemolymph space and also adds to the driving force for the generation of the sensillar potential (Thurm and Wessel 1979). Both ORNs send axons through the antennal nerve to the next higher neuropil, the antennal lobe (Sanes and Hildebrand 1976).

Insect olfactory transduction

Before insect olfactory receptors were cloned, heterologously expressed and electrophysiologically characterized, most investigations on olfactory transduction processes in insect olfactory sensilla supported the hypothesis that the transduction is mainly mediated by G-protein coupled signalling cascades. It was shown that the activity of phospholipase C in antennal membranes of *Periplaneta americana* and *Locusta migratoria* was stimulated by application of odorants and pheromones (Boekhoff et al. 1990a,b). The stimulatory effect was enhanced with GTP analogues and attenuated with GDP analogues, whereas the application of pertussis toxin completely blocked the effect, indicating a G_o- or G_i- mediated reaction (Boekhoff et al. 1990a). Furthermore, the levels of inositol-1,4,5-triphosphate (IP_3) increased rapidly after pheromone stimulation in antennal preparations of *Heliothis virescens* and *P. americana* (Breer et al.

1990; Boekhoff et al. 1990b; Boekhoff et al. 1993). In addition, IP_3-dependent Ca^{2+}-currents were characterized in patch clamp recordings of cultured ORNs of *M. sexta*, which triggered the opening of Ca^{2+}-dependent ion channels involved in the generation of pheromone-dependent receptor potentials (Zufall et al. 1991; Stengl 1993, 1994). The following concept for the signal transduction in pheromone-sensitive ORNs was developed: The binding of odorants to its specific receptors activate G-proteins, which then stimulate phospholipase C. This leads to the transient generation of IP_3 and diacylglycerol (DAG), followed by the IP_3-dependent opening of Ca^{2+}-channels. The following increase in the intracellular Ca^{2+}-concentration induces the opening of Ca^{2+}-dependent unspecific cation channels, further increasing intracellular Ca^{2+}-levels. The Ca^{2+}-dependent cation channels close due to auto-adaptation when Ca^{2+}-levels reach a specific threshold. The DAG-dependent activation of a protein kinase C, which phosphorylates more unspecific cation channels, is assumed to occur only after long and strong pheromone stimuli (Stengl 1999).

In contrast, more recent studies proposed that G-proteins only play a minor role in insect olfactory transduction. The first step to a novel model of the primary signal transduction was the cloning and identification of insect olfactory receptors (OR) in *Drosophila melanogaster* (Vosshall et al. 1999; Clyne et al. 1999) and later in moth species, like *Bombyx mori* (Sakurai et al. 2004) and *M. sexta* (Patch et al. 2009). It was shown that, apart from various odour ligand-binding olfactory receptors, ubiquitous chaperone proteins (OR83b for *D. melanogaster*, BmOR2 for *B. mori* and MsextaOR2 for *M. sexta*) exist, which are expressed in the majority of ORNs and which are highly conserved among insect species. The non-odour-sensitive OR83b has a structure which is similar to vertebrate G-protein-coupled-receptors, but has a reversed membrane topology with the C terminus located extracellularly (Benton et al. 2006) and is responsible for the localization of other tuning ORs in the dendritic membrane (Larsson et al. 2004). OR83b and its analogues do not function in odour recognition but form heteromeric complexes with functional ORs, highly enhancing their sensitivity to odour stimulation (Neuhaus et al. 2005). This was also shown for combinations of pheromone-sensitive ORs with the OR83b analogue BmOR2 in *B. mori* (Nakagawa et al. 2005). Most striking were patch clamp recordings from heterologously expressed ORs with OR83b which revealed that the receptor

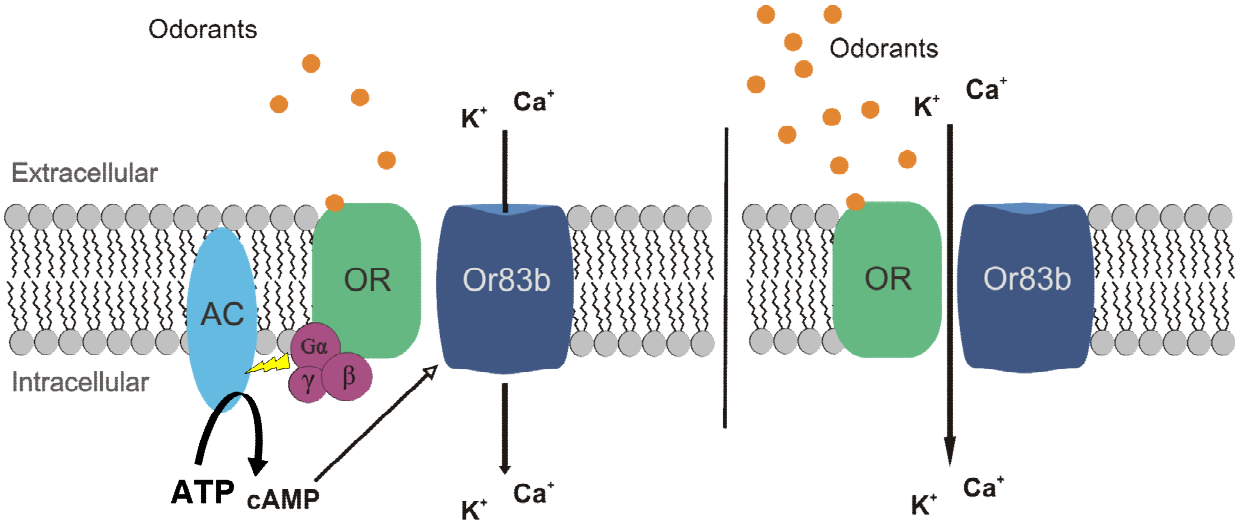

Fig. 2: Hypothetical model of the odorant-dependent activation of cation channels in insect ORNs of *D. melanogaster* (**a**): After stimulation with low odorant concentrations the functional olfactory receptor triggers the activation of an adenylyl cyclase leading to slow increases in cAMP-levels. cAMP then activates the cyclic-nucleotide sensitive non-selective cation channel OR83b. (**b**) During high odorant concentrations the receptor complex, which forms non-specific cation channels consisting of the functional OR and OR83b, is directly activated by the binding of odorant molecules, leading to low latency K^+- and Ca^{2+}-inward currents.

complexes act as direct ligand-gated non-selective cation channels (Sato et al. 2008; Wicher et al. 2008). In contrast, Wicher et al. (2008) also observed a slow but strong metabotropic component of the receptor current which relied on intracellular ATP and GTP. After stimulation of OR22a a G-protein mediated increase in the cAMP levels by activation of an adenylyl cyclase was found. In addition, it was shown that OR83b is activated by cyclic nucleotides and that it forms a non-selective cation channel (Wicher et al. 2008). The application of G-protein inhibitors blocked the odour-induced cAMP production and shifted the dose response curve to higher odorant doses. It was assumed that strong pheromone responses are mediated by the activation of the directly ligand-gated ion channel complex and that only after weak odour stimulation the G-protein coupled pathway is activated to amplify the perceived odour signal (Fig. 2). Due to the late activation of the cAMP-dependent current after the stimulation and the restriction of inhibiting effects on the rectification of the receptor signal (Smart et al. 2008), it is likely that the cAMP-dependent current is rather involved in the modulation of the odour transduction than in the initial generation of the receptor current. It also remains to be shown whether these mechanisms also contribute to odour transduction *in vivo* and whether additional or other mechanisms are present in pheromone sensitive ORNs of moth species.

Role of cyclic nucleotides and biogenic amines in insect olfactory transduction

Cyclic guanosine monophosphate (cGMP) is supposed to play an important role in long-term adaptation of ORNs of both invertebrates and vertebrates. It was shown that cGMP levels rise slowly after strong pheromone stimulation in antennae and antennal homogenates of *Antheraea polyphemus* and *B. mori* (Ziegelberger et al. 1900; Boekhoff et al. 1993). The addition of exogenous cGMP during pheromone stimulation strongly decreased the phasic component of the IP_3 increase (Boekhoff et al. 1993), suggesting a cGMP dependent decrease in the phasic response of ORNs during adaptation (Kodadová 1996). In addition, the perfusion of the sensillar lymph with a cGMP-analogue decreased the sensillar potential amplitude and action potential frequency in tip recordings of pheromone-sensitive sensilla of the silkmoth *B. mori* (Redkozubov 2000). In immunocytochemical experiments it was found that in a subpopulation of ORNs of male *M. sexta* minute long stimulation with pheromones increased intracellular cGMP concentrations (Stengl et al. 2001). These increases were synergistically enhanced by the activation of a pheromone-inducible nitric oxide-synthase-like enzyme in ORNs (Stengl and Zintl 1996; Stengl et al. 2001). Also in vertebrates cGMP is involved in the long-term adaptation of ORNs (Zufall and Leinders-Zufall 2000), whereas cGMP activates cyclic nucleotide sensitive channels which leads to an inward Ca^{2+}-current. Increased Ca^{2+}-levels then affect targets in the G-protein coupled transduction pathway attenuating following responses to odour stimulation.

In contrast, cyclic adenosine monophosphate (cAMP) is assumed to take part in the sensitization of insect ORNs. While relatively much is known about the role of cAMP in vertebrate olfaction, in which cAMP constitutes the main second messenger in the G-protein coupled pathway leading to excitation of the ORNs (Nakamura 2000), less is known about the function of cAMP in invertebrate olfaction. In lobster ORNs dual second messenger pathways were found (Boekhoff et al. 1994; Hatt and Ache 1994), whereas the hyperpolarization observed after stimulation with specific odours is mediated by increases in the level of intracellular cAMP and the subsequent activation of cyclic nucleotide gated channels. To our knowledge only a single study investigated the influence of cAMP on the pheromone transduction in a moth species. Villet (1978) showed that the perfusion of antennae of *Antheraea pernyi* with cAMP or phosphodiesterase inhibitors increased electroantennograms (EAGs) recorded in response to pheromone stimulation. In addition, the overexpression of a cAMP-phosphodiesterase in olfactory organs of *D. melanogaster* affected the behavioural response to odours (Gomez-Diaz et al. 2004) and EAG onset kinetics (Martín et al. 2001). As mentioned before, the investigation of currents of heterologously expressed *D. melanogaster* receptor complexes also revealed a contribution of cAMP in a possible amplification of weak odour signals (Wicher et al. 2008). However, this cAMP-dependent activation of OR83b could also be involved in the modulation of the odour transduction cascade.

Furthermore octopamine, acting as neuromodulator, neurohormone and neuromediator was found to have sensitizing effects on ORNs of different insect species. In tip recordings of *B. mori* the injection of octopamine into the hemolymph was followed by increases in the pheromone-dependent sensillar potential and action potential response (Pophof 2002), suggesting a direct effect on the periphery of the olfactory neural pathway. Also in *A. polyphemus*, *Mamestra brassicae* and *P. americana* octopamine sensitized ORNs but only on the level of the action potential response (Pophof 2000; Grosmaitre et al. 2001; Zhukovskaya and Kapitsky 2006). In addition, octopamine was effective in behavioural experiments, since the responsiveness of male moths to pheromone was enhanced after octopamine application (Linn Jr and Roelofs 1986, 1992; Linn Jr et al. 1992). Aside from the disadapting effects on various sensory systems octopamine is also involved in associative olfactory learning and memory consolidation (Hammer and Menzel 1998; Schwaerzel et al. 2003; Farooqui et al. 2003). Octopamine in insects originates mainly from octopaminergic dorsal and ventral unpaired median neurons which send processes to most parts of the brain and to the periphery (Roeder 2005; Farooqui 2007). In *Apis mellifera* and *M. sexta* ventral unpaired median neurons project to the antennal lobes, to the calyces of the mushroom body and to the lateral protocerebrum, which are involved in the processing of olfactory information (Schröter et al. 2007; Dacks et al. 2005). The innervation pattern of possible octopaminergic neurons in the antennae is mostly unknown. Octopamine receptors and octopamine/tyramine receptors, which show a higher affinity to the octopamine precursor tyramine, were cloned and characterized in several insect species (Balfanz et al. 2005; Farooqui 2007), whereas only three of them were shown to be expressed in antennae and in the trichoid sensilla of moths (Krieger et al. 1999; Dacks et al. 2006; Brigaud et al. 2009). Octopamine receptors are G-protein coupled receptors which mostly activate adenylyl cyclases and also upregulate intracellular Ca^{2+}-concentrations (Farooqui 2007). While the majority of octopamine-alpha-adrenergic-like receptors are positively coupled to adenylyl cyclases (Evans and Maqueira 2005; Farooqui 2007), octopamine/tyramine receptors were found to inhibit adenylyl cyclases (Krieger et al. 1999; Farooqui 2007) and therefore mostly show opposite effects to octopamine. It is likely that the observed octopamine effects are partly due to increases in the intracellular cAMP-concentration and its action on cyclic nucleotide sensitive targets, like the cAMP-sensitive binding site on OR83b or cyclic nucleotide sensitive ion channels.

Circadian rhythms in insect olfaction

The pheromone production, pheromone release, calling behaviour of female moths and also the male responsiveness to pheromone expresses circadian diurnal rhythms in many moth species (Baker and Cardé 1979; Choi et al. 1998; Delisle and Simard 1999). The pheromone production of female and the pheromone responsiveness of male *Agrotis segetum* are regulated endogenously by a circadian clock, since both rhythms with maxima at the end of the scotophase persisted under constant conditions (Rosén 2002; Rosén et al. 2003). Furthermore, males and females of *Spodoptera littoralis* raised in light:dark cycles out of phase mated less frequently and pre-exposure to pheromone in constant darkness increased pheromone responsiveness of male moth. This suggests that not only the photoperiod but also the female pheromone signal acts as a Zeitgeber, synchronizing rhythms in male and female moths (Silvegren et al. 2005). Also the circadian rhythm in the calling behaviour of female *M. sexta* showed a diurnal distribution with a maximum at the end of the scotophase (Itagaki and Conner 1988), correlating with peaks in male flight activity (Sasaki and Riddiford 1984).

Octopamine, the so called "stress hormone" of insects, modulates the fat body glycogenolysis and therefore is highly important for the energy supply during high flight activity occurring during the scotophase of nocturnal moths (Roeder 2005). Furthermore, octopamine was shown to desensitize inputs in various sensory systems (Roeder 2005; Farooqui 2007). It is assumed that octopamine plays a key role in the circadian regulation of circadian rhythms in male responsiveness to pheromone and also in the pheromone production. For *M. sexta* and for *Trichoplusia ni* circadian changes in the concentration of octopamine in the hemolymph and brain were found, which correlated with rhythms in the pheromone responsiveness

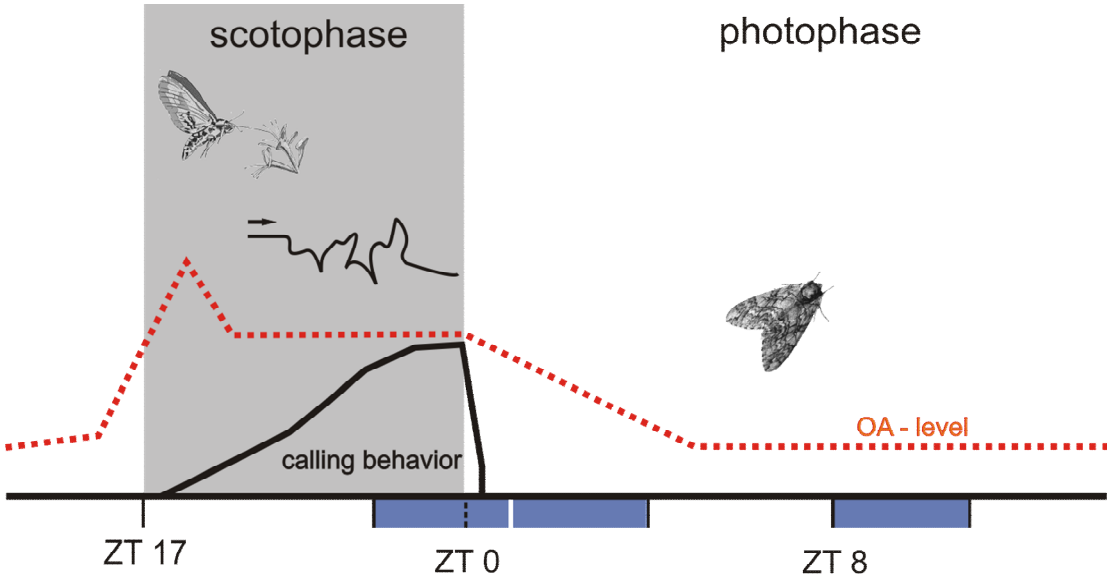

Fig. 3: Schematic and idealized diagram of photoperiod-dependent differences in the behaviour and putative octopamine concentrations in the hemolymph of *Manduca sexta*. The animals were kept under long-day photoperiod conditions (L:D 17:7h) with Zeitgebertime (ZT) 0 = lights on. In the scotophase when feeding and mating behaviours occur, octopamine levels were shown to be high. The calling behaviour of female moths begins in the first third of the scotophase and reaches its maximum at the end of the scotophase. With beginning of the photophase moths switch from their active to their inactive phase and octopamine levels start to decline. During the middle of the photophase most moths rest and octopamine levels are low. The experiments in this study were performed during three different Zeitgebertimes (blue squares: ZT 22-1, ZT 1-4 and ZT 8-11).

(Lehman 1990; Linn Jr et al. 1994, 1996). Concentrations of octopamine in the hemolymph were shown to be high during the scotophase, when both mating and feeding behaviours occur and low during the photophase when the nocturnal moths rest (Fig. 3). In addition, the sensitizing effect of octopamine on the responsiveness to pheromone was shown to be time-dependent, since octopamine was only effective when it was injected during low hemolymph octopamine concentrations in the photophase but not during the scotophase (Linn Jr and Roelofs 1992; Linn Jr et al. 1992).

One main question in the research of circadian regulation of pheromone sensitivity during the last years was, whether the sensitivity is controlled at the periphery or on higher located neuropils in the olfactory neuronal pathway. In contrast to the hierarchical architecture of the mammalian circadian system, where light entrains a central oscillator located in the suprachiasmatic nucleus, that then entrains pacemakers in peripheral tissues, oscillators in different insect tissues were shown to operate independently from each other (Plautz et al. 1997). Furthermore, it was unknown whether circadian pacemakers also exist in the antennae directly controlling the observed rhythms. While the knowledge about circadian processes at the periphery for moth species is still incomplete much is known about the regulation of circadian olfactory rhythms in the fruit fly. In EAG recordings from antennae of *D. melanogaster* circadian rhythms were found to stimulation with non-pheromone odorants, which were abolished in mutants lacking the clock genes *per* and *tim* (Krishnan et al. 1999). These rhythms are controlled by pacemakers in the antennae and the peripheral circadian clock neurons were shown to be sufficient and necessary for the generation of olfactory rhythms in *D. melanogaster* (Tanoue et al. 2004). This was also shown on the behavioural level (Zhou et al. 2005). More recent studies also proved that olfactory receptors are rhythmically accumulated in the dendrites of ORNs and that this accumulation is dependent on the rhythmic expression of the G-protein-coupled receptor kinase 2 (GPRK2) (Tanoue et al. 2008). In contrast, in recordings from single sensilla no circadian rhythms were found in responses to odour and pheromone stimulation or in the

spontaneous activity (Krishnan et al. 2008). Only in non-pheromone sensitive basiconic sensilla a GPRK2-dependent rhythm in the action potential amplitude was found, whose physiological relevance is unknown (Krishnan et al. 2008). In moths the data on pheromone-dependent olfactory rhythms is fragmentary. In EAG recordings from *Spodoptera littoralis* a circadian rhythm was found in responses to pheromone stimulation, although it is in contradiction to the behaviour of the moths (Merlin et al. 2007). Furthermore, rhythms in the expression of several clock genes and of a pheromone degrading enzyme in the antennae were observed. These results suggest that also in antennae of moth species peripheral oscillators exist, that might be involved in the regulation of olfactory circadian rhythms. The PERIOD-like staining in ORNs and in accessory cells of pheromone-sensitive trichoid sensilla of *M. sexta* (Schuckel et al. 2007), together with strong time-dependent differences in the expression of per messenger-RNA supports this hypothesis. To elucidate time-dependent differences in the sensitivity of pheromone sensitive trichoid sensilla and to elucidate time-dependent effects of biogenic amines and cyclic nucleotides, the experiments in this study were performed during three different Zeitgebertimes (Fig. 3).

Resolution of pulsed pheromone signals

Not only the absolute pheromone concentration, but also the distribution of the pheromone signal in space and time is important information for the males in finding the source of pheromone emission. Female moths emit pulsed pheromone signals by rhythmic extrusion and retraction of the terminal abdominal segments (Itagaki and Conner 1985, 1988; Crnjar et al. 1988; Valeur et al. 1999), where the pheromone glands are located (Eaton 1986; Itagaki and Conner 1988). In addition, under natural conditions where turbulence dominates over diffusion the pulsed signal is further fragmented into filaments of various scale and frequency. Thus, naturally the pheromone signal is not a linear gradient of pheromone concentrations decreasing with distance from the source, but is a highly intermittent signal. During evolution the olfactory system on the peripheral and central level adapted to the special characteristics of this signal. ORNs of *M. sexta* are able to encode intermittent pheromone signals up to a frequency of 3 Hz (Marion-Poll and Tobin 1992). Also in other moths ORNs were shown to be able to encode pulsed pheromone signals (Almaas et al. 1991; Kodadová 1996). Furthermore, in EAG recordings from antennae from several moth species evidence was provided that some components of the antennae are able to follow pheromone signals with frequencies between 25 and 33 Hz (Bau et al. 2002, 2005). Also neurons in the antennal lobe of *M. sexta* are able to follow pulsed pheromone signals (Christensen and Hildebrand 1988; Christensen et al. 1989). How important the intermittency of the pheromone signal for the successful search behaviour for male moths is, was also shown in wind tunnel experiments. Males of *Heliothis virescens* did not locate the pheromone source in significant numbers when the pheromone was presented in a continuous plume, but only when the frequency of filaments exceeded 4 Hz (Vickers and Baker 1992). This was also shown for *Grapholita molesta*, which only sustained upwind flight when the signal was pulsed, but not when it was provided as a continuous plume (Baker et al. 1985). Furthermore, shifts in the fine structure of the pheromone plume including more turbulent airstreams had a stronger impact on the flight patterns of male *Cadra cautella* than a 1000-fold increase in the pheromone dosage (Mafra-Neto and Cardé 1995). The ability of peripheral ORNs to encode pulsed pheromone signals is mainly determined by the distribution of action potentials in responses to pheromone stimulation. ORNs with more phasic responses are able to encode higher frequencies than ORNs with a tonic action potential distribution (Almaas et al. 1991; Kodadová 1996). Thus, the ability of male moths to find pheromone sources is mainly determined by the pulse resolution of peripheral ORNs and therefore is highly important for the successful search behaviour.

List of abbreviations:

BAL	Bombykal
cAMP	3'-5'-cyclic adenosine monophosphate
cGMP	3'-5'-cyclic guanosine monophosphate
DAG	Diacylglycerol
EAG	Electroantennogram
GPRK2	G-protein-coupled receptor kinase 2
IP_3	Inositol-1,4,5-triphosphate
OR	Olfactory receptor
ORN	Olfactory receptor neuron

References:

Almaas TJ, Christensen TA, Mustaparta H (1991) Chemical communication in heliothine moths: I. Antennal receptor neurons encode several features of intra- and interspecific odorants in the male corn earworm moth *Helicoverpa zea*. J Comp Physiol A 169:249-258

Baker TC, Cardé RT (1979) Endogenous and exogenous factors affecting periodicities of female calling and male sex pheromone response in *Grapholitha molesta* (Busck). J Insect Physiol 25:943-950

Baker TC, Willis MA, Haynes KF, Phelan PL (1985) A pulsed cloud of sex pheromone elicits upwind flight in male moths. Physiol Entomol 10:257-265

Balfanz S, Strünker T, Frings S, Baumann A (2005) A family of octopamine [corrected] receptors that specifically induce cyclic AMP production or Ca^{2+} release in *Drosophila melanogaster*. J Neurochem 93:440-451 (Erratum: J Neurochem 94:1168, 2005)

Bau J, Justus KA, Cardé RT (2002) Antennal resolution of pulsed pheromone plumes in three moth species. J Insect Physiol 48:433-442

Bau J, Justus KA, Loudon C, Cardé RT (2005) Electroantennographic resolution of pulsed pheromone plumes in two species of moths with bipectinate antennae. Chem Senses 30:771-780

Benton R, Sachse S, Michnick SW, Vosshall LB (2006) Atypical membrane topology and heteromeric function of *Drosophila* odorant receptors in vivo. PLoS Biol 4:e20.

Boekhoff I, Raming K, Breer H (1990a) Pheromone-induced stimulation of inositol-trisphosphate formation in insect antennae is mediated by G-proteins. J Comp Physiol 160, 99-103

Boekhoff I, Michel WC, Breer H, Ache BW (1994) Single odors differentially stimulate dual second messenger pathways in lobster olfactory receptor cells. J Neurosci 14:3304-3309

Boekhoff I, Strotmann J, Raming K, Tareilus E, Breer H (1990b) Odorant-sensitive phospholipase C in insect antennae. Cell Signal 2:49-56

Boekhoff I, Seifert E, Göggerle S, Lindemann M, Krüger BW, Breer H (1993) Pheromone-induced second-messenger signaling in insect antennae. Insect Biochem Molec Biol 23:757-762

Breer H, Boekhoff I, Tareilus E (1990) Rapid kinetics of second messenger formation in olfactory transduction. Nature 345:65-68

Brigaud I, Grosmaître X, François MC, Jacquin-Joly E (2009) Cloning and expression pattern of a putative octopamine/tyramine receptor in antennae of the noctuid moth *Mamestra brassicae*. Cell Tissue Res 335:455-463

Choi MY, Tatsuki S, Boo KS (1998) Regulation of sex pheromone biosynthesis in the oriental tobacco budworm, *Helicoverpa assulta* (Lepidoptera: Noctuidae). J Insect Physiol 44:653–658

Christensen TA, Hildebrand JG (1988) Frequency coding by central olfactory neurons in the sphinx moth *Manduca sexta*. Chem. Senses 13, 123-130

Christensen TA, Mustaparta H, Hildebrand JG (1989). Discrimination of sex pheromone blends in the olfactory system of the moth. Chem Senses 14:463-477

Clyne PJ, Warr CG, Freeman MR, Lessing D, Kim J, Carlson JR (1999) A novel family of divergent seven-transmembrane proteins: candidate odorant receptors in *Drosophila*. Neuron 22:327-338

Crnjar R, Angioy AM, Pietra P, Yin CM, Liscia A, Barbarossa IT (1988) Control mechanisms of calling behaviour in *Lymantria dispar*: An electrophysiological investigation on the role of the terminal abdominal ganglion. J Insect Physiol 34:1087-1091

Dacks AM, Dacks JB, Christensen TA, Nighorn AJ (2006) The cloning of one putative octopamine receptor and two putative serotonin receptors from the tobacco hawkmoth *Manduca sexta.* Insect Biochem Mol Biol 36:741-747

Dacks AM, Christensen TA, Agricola HJ, Wollweber L, Hildebrand JG (2005) Octopamine-immunoreactive neurons in the brain and subesophageal ganglion of the hawkmoth *Manduca sexta*. J Comp Neurol 488:255-268

Delisle J, Picimbon J-F, Simard J (1999) Physiological control of pheromone production in *Choristoneura fumiferana* and *C. Rosaceana*. Arch Insect Biochem Physiol 42:253-268

Dolzer J, Krannich S, Fischer K, Stengl M (2001) Oscillations of the transepithelial potential of moth olfactory sensilla are influenced by octopamine and serotonin. J Exp Biol 204:2781-2794

Eaton JL (1986) Morphology of abdominal segments eight and nine of the female tobacco hornworm, *Manduca sexta* (Lepidoptera: Sphingidae). Ann Entomol Soc Am 79:629-635

Evans PD, Maqueira B (2005) Insect octopamine receptors: a new classification scheme based on studies of cloned *Drosophila* G-protein coupled receptors. Invert Neurosci 5:111-118

Farooqui T (2007) Octopamine-mediated neuromodulation of insect senses. Neurochem Res 32:1511-1529

Farooqui T, Robinson K, Vaessin H, Smith BH (2003) Modulation of early olfactory processing by an octopaminergic reinforcement pathway in the honeybee. J Neurosci 23:5370-5380

Gomez-Diaz C, Martin F, Alcorta E (2004). The cAMP transduction cascade mediates olfactory reception in *Drosophila melanogaster*. Behav Genet 34:395-406

Grosmaitre X, Marion-Poll F, Renou M (2001) Biogenic amines modulate olfactory receptor neurons firing activity in *Mamestra brassicae*. Chem Senses 26:653-661

Hammer M, Menzel R (1998) Multiple Sites of Associative Odor Learning as Revealed by Local Brain Microinjections of Octopamine in Honeybees. Learn Mem 5:146–156

Hatt H, Ache BW (1994) Cyclic nucleotide- and inositol phosphate-gated ion channels in lobster olfactory receptor neurons. Proc Natl Acad Sci USA 91:6264-6268

Itagaki H, Conner WE (1985) Physiological control of pheromone release behaviour in *Manduca sexta* (L.). J Insect Physiol 32:657:664

Itagaki H, Conner WE (1988) Calling Behavior of *Manduca sexta* (L.) (Lepidoptera: Sphingidae) with Notes on the Morphology of the Female Sex Pheromone Gland. Ann Ent. Soc Am 81:798-807

Kalinová B, Hoskovec M, Liblikas I, Unelius CR, Hansson BS (2001) Detection of sex pheromone components in *Manduca sexta* (L.). Chem Senses 26:1175-1186

Keil T (1989) Fine structure of the pheromone-sensitive sensilla on the antenna of the hawkmoth, *Manduca sexta*. Tissue Cell 21:139-151

Kodadová B (1996). Resolution of pheromone pulses in receptor cells of *Antheraea polyphemus* at different temperatures. J Comp Physiol A 179:301-310

Krieger J, Strobel J, Vogl A, Hanke W, Breer H (1999) Identification of a cyclic nucleotide- and voltage-activated ion channel from insect antennae. Insect Biochem Mol Biol 29:255-267

Krishnan B, Dryer SE, Hardin PE (1999) Circadian rhythms in olfactory responses of *Drosophila melanogaster*. Nature 400:375-378

Krishnan P, Chatterjee A, Tanoue S, Hardin PE (2008) Spike amplitude of single-unit responses in antennal sensillae is controlled by the *Drosophila* circadian clock. Curr Biol 18:803-807

Larsson MC, Domingos AI, Jones WD, Chiappe ME, Amrein H, Vosshall LB (2004) Or83b encodes a broadly expressed odorant receptor essential for *Drosophila* olfaction. Neuron 43:703-714

Lee JK, Strausfeld NJ (1990) Structure, distribution, and number of surface sensilla and their receptor cells on the antennal flagellum of the male sphinx moth *Manduca sexta*. J Neurocytol 19:519-538

Lehman HK (1990) Circadian control of *Manduca sexta* flight. Abstr Soc Neurosci 16:1334

Linn CE Jr, Roelofs WL (1986) Modulatory effects of octopamine and serotonin on male sensitivity and periodicity of response to sex pheromone in the cabbage looper moth, *Trichoplusia ni*. Arch Insect Biochem Physiol 3:161-171

Linn CE Jr, Roelofs WL (1992) Role of photoperiod cues in regulating the modulatory action of octopamine on pheromone-response thresholds in the cabbage looper moth. Arch Insect Biochem Physiol 20:285-302

Linn CE Jr, Campbell MG, Roelofs WL (1992) Photoperiod cues and the modulatory action of octopamine and 5-hydroxytryptamine on locomotor and pheromone response in male gypsy moths, *Lymantria dispar*. Arch Insect Biochem Physiol 20:265-284

Linn CE Jr, Poole KR, Roelofs WL (1994) Studies on biogenic amines and their metabolites in nervous tissue and hemolymph of adult male cabbage looper moths-I. Quantitation of photoperiod changes. Comp Biochem Physiol 108:73-85

Linn CE Jr, Campbell MG, Poole KR, Wu W-Q, Roelofs WL (1996) Effects of photoperiod on the circadian timing of pheromone response in male *Trichoplusia ni*: Relationship to the modulatory action of octopamine. J Insect Physiol 42:881-891

Mafra-Neto A, Cardé RT (1995) Influence of plume structure and pheromone concentration on upwind flight of *Cadra cautella* males. Physiol Entomol 20:117-133

Marion-Poll F, Tobin TR (1992) Temporal coding of pheromone pulses and trains in *Manduca sexta*. J Comp Physiol A 171:505-512

Martín F, Charro MJ, Alcorta E (2001) Mutations affecting the cAMP transduction pathway modify olfaction in *Drosophila*. J Comp Physiol A 187:359-370

Merlin C, Lucas P, Rochat D, François MC, Maibèche-Coisne M, Jacquin-Joly E (2007) An antennal circadian clock and circadian rhtyhms in peripheral pheromone reception in the Moth *Spodoptera littoralis*. J Biol Rhythms 22:502-514

Nakagawa T, Sakurai T, Nishioka T, Touhara K (2005) Insect sex-pheromone signals mediated by specific combinations of olfactory receptors. Science 307:1638-1642

Nakamura T (2000) Cellular and molecular constituents of olfactory sensation in vertebrates. Comp Biochem Physiol A 126:17-32

Neuhaus EM, Gisselmann G, Zhang W, Dooley R, Störtkuhl K, Hatt H (2005) Odorant receptor heterodimerization in the olfactory system of *Drosophila melanogaster*. Nat Neurosci 8:15-17

Patch HM, Velarde RA, Walden KK, Robertson HM (2009) A Candidate Pheromone Receptor and Two Odorant Receptors of the Hawkmoth *Manduca sexta*. Chem Senses doi:10.1093/chemse/bjp002

Plautz JD, Kaneko M, Hall JC, Kay SA (1997) Independent photoreceptive circadian clocks throughout *Drosophila*. Science 278:1632-1635

Pophof B (2000) Octopamine modulates the sensitivity of silkmoth pheromone receptor neurons. J Comp Physiol A 186:307-313

Pophof B (2002) Octopamine enhances moth olfactory responses to pheromones, but not those to general odorants. J Comp Physiol A 188:659-662

Redkozubov A (2000) Guanosine 3′,5′-cyclic monophosphate reduces the response of the Moth's olfactory receptor neuron to pheromone. Chem Senses 25:381-385

Roeder T (2005) Tyramine and octopamine: ruling behavior and metabolism. Annu Rev Entomol 50:447-477

Rosén WQ (2002) Endogenous control of circadian rhythms of pheromone production in the turnip moth *Agrotis segetum*. Arch Insect Biochem Physiol 50:21-30

Rosén WQ, Han GB, Löfstedt C (2003) The circadian rhythm of the sex-pheromone-mediated behavioral response in the turnip moth, *Agrotis segetum*, is not controlled at the peripheral level. J Biol Rhythms 18:402-408

Sakurai T, Nakagawa T, Mitsuno H, Mori H, Endo Y, Tanoue S, Yasukochi Y, Touhara K, Nishioka T (2004) Identification and functional characterization of a sex pheromone receptor in the silkmoth *Bombyx mori*. Proc Natl Acad Sci USA 101:16653-16658

Sanes JR, Hildebrand JG (1976) Structure and development of antennae in a moth, *Manduca sexta*. Dev Biol 51:280-299

Sasaki M, Riddiford LM (1984) Regulation of reproductive behaviour and egg maturation in the tobacco hawk moth, *Manduca sexta.* Physiol Entomol 9:315-327

Sato K, Pellegrino M, Nakagawa T, Nakagawa T, Vosshall LB, Touhara K (2008) Insect olfactory receptors are heteromeric ligand-gated ion channels. Nature 452:1002-1006

Schröter U, Malun D, Menzel R (2007) Innervation pattern of suboesophageal ventral unpaired median neurones in the honeybee brain. Cell Tissue Res 327:647-667

Schuckel J, Siwicki KK, Stengl M (2007) Putative circadian pacemaker cells in the antenna of the hawkmoth *Manduca sexta*. Cell Tissue Res 330:271-278

Schwaerzel M, Monastirioti M, Scholz H, Friggi-Grelin F, Birman S, Heisenberg M (2003) Dopamine and octopamine differentiate between aversive and appetitive

olfactory memories in *Drosophila*. J Neurosci 23:10495-10502

Silvegren G, Löfstedt C, Rosén WQ (2005) Circadian mating activity and effect of pheromone pre-exposure on pheromone response rhythms in the moth *Spodoptera littoralis*. J Insect Physiol 51:277-286

Smart R, Kiely A, Beale M, Vargas E, Carraher C, Kralicek AV, Christie DL, Chen C, Newcomb RD, Warr CG (2008) *Drosophila* odorant receptors are novel seven transmembrane domain proteins that can signal independently of heterotrimeric G proteins. Insect Biochem Mol Biol 38:770-780

Stengl M (1993) Intracellular-messenger-mediated cation channels in cultured olfactory receptor neurons. J Exp Biol 178:125-147

Stengl M (1994) Inositol-trisphosphate-dependent calcium currents precede cation currents in insect olfactory receptor neurons in vitro. J Comp Physiol A 174:187-194

Stengl M, Zintl R (1996) NADPH-diaphorase staining in the antenna of the moth *Manduca sexta*. J Exp Biol 199:1063-1072

Stengl M, Ziegelberger G, Boekhoff I, Krieger J (1999) Perireceptor events and transduction mechanisms in insect olfaction. In *Insect olfaction* (ed. B. S.Hansson BS), pp. 49-66. Berlin: Springer Verlag.

Stengl M, Zintl R, de Vente J, Nighorn A (2001) Localization of cGMP-immunoreactivity and of soluble guanylyl cyclase in antennal sensilla of the hawkmoth *Manduca sexta*. Cell and Tissue Res 304:409-421

Tanoue S, Krishnan P, Chatterjee A, Hardin PE (2008) G protein-coupled receptor kinase 2 is required for rhythmic olfactory responses in *Drosophila*. Curr Biol 18:787-794

Tanoue S, Krishnan T, Krishnan B, Dryer SE, Hardin PE (2004) Circadian clocks in antennal neurons are necessary and sufficient for olfaction rhythms in *Drosophila*. Curr Biol 14:638-649

Thurm U, Wessel G (1979) Metabolism-dependent transepithelial potential differences at epidermal receptors of arthropods. I. Comparative data. J Comp Physiol 134:119-130

Valeur PG, Hansson BS, Löfstedt C (1999) Real-time measurement of pheromone release from individual female moths and synthetic dispensers in a wind tunnel by recording of single receptor-neuron responses. Physiol Entomol 24:240-250

Vickers NJ, Baker TC (1992) Male *Heliothis virescens* maintain upwind flight in response to experimentally pulsed filaments of their sex pheromone (Lepidoptera: Noctuidae). J Insect Behav 5:669-687

Villet RH (1978) Mechanism of insect sex-pheromone sensory transduction: role of adenylate cyclase. Comp Biochem Physiol 61:389-394

Vosshall LB, Amrein H, Morozov PS, Rzhetsky A, Axel R (1999) A spatial map of olfactory receptor expression in the *Drosophila* antenna. Cell 96:725-736

Wicher D, Schäfer R, Bauernfeind R, Stensmyr MC, Heller R, Heinemann SH, Hansson BS (2008) *Drosophila* odorant receptors are both ligand-gated and cyclic-nucleotide-activated cation channels. Nature 452:1007-1011

Wieczorek H, Putzenlechner M, Zeiske W, Klein U (1991). A vacuolar-type proton pump energizes K^+/H^+ antiport in an animal plasma membrane. J Biol Chem 266:15340-15347

Wieczorek H, Huss M, Merzendorfer H, Reineke S, Vitavska O, Zeiske W (2003) The insect plasma membrane H^+ V-ATPase: intra-, inter-, and supramolecular aspects. J Bioenerg Biomembr 35:359-366

Zhou X, Yuan C, Guo A (2005) *Drosophila* olfactory response rhythms require clock genes but not pigment dispersing factor or lateral neurons. J Biol Rhythms 20:237-244

Zhukovskaya MI, Kapitsky SV (2006) Activity modulation in cockroach sensillum: the role of octopamine. J Insect Physiol 52:76-86

Ziegelberger G, van den Berg MJ, Kaissling KE, Klumpp S, Schultz JE (1990) Cyclic GMP levels and guanylate cyclase activity in pheromone-sensitive antennae of the silkmoths *Antheraea polyphemus* and *Bombyx mori*. J Neurosci 10:1217-1225

Zufall F, Leinders-Zufall T (2000) The cellular and molecular basis of odor adaptation. Chem Senses 25:473-481

Zufall F, Stengl M, Franke C, Hildebrand JG, Hatt H (1991) Ionic currents of cultured olfactory receptor neurons from antennae of male *Manduca sexta*. J Neurosci 11: 956-965

Perfusion with cGMP analogue adapts the action potential response of pheromone-sensitive sensilla trichoidea of the hawkmoth *Manduca sexta* in a daytime-dependent manner

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Perfusion with cGMP analogue adapts the action potential response of pheromone-sensitive sensilla trichoidea of the hawkmoth *Manduca sexta* in a daytime-dependent manner

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Summary

Pheromone-dependent mate search is under strict circadian control in different moth species. But it remains unknown whether daytime-dependent changes in pheromone sensitivity already occur at the periphery in male moths. Because adapting pheromone stimuli cause rises of cyclic guanosine monophosphate (cGMP) in pheromone-sensitive trichoid sensilla of the night-active hawkmoth *Manduca sexta*, we wanted to determine whether cGMP decreases pheromone-sensitivity of olfactory receptor neurons in a daytime-dependent manner. Long-term tip recordings from trichoid sensilla were performed at the early day (ZT 1–4), when many moths are still active, and at the middle of the day (ZT 8–11), when moths are resting. A non-adapting pheromone-stimulation protocol combined with perfusion of the sensillum lymph with the membrane-permeable cGMP analogue 8bcGMP adapted the action potential response but not the sensillar potential. Perfusion with 8bcGMP decreased the initial action potential frequency, decreased the numbers of action potentials elicited in the first 100 ms of the pheromone response and attenuated the reduction of action potential amplitude. Furthermore, the decrease in 8bcGMP-dependent action potential frequency was stronger in recordings made at ZT 8–11 than at ZT 1–4. In the control recordings during the course of the day the pheromone responses became increasingly tonic and less phasic. At ZT 8–11 only, this daytime-dependent effect was further enhanced by 8bcGMP application. Thus we hypothesize that during the moths' resting phase, elevated cGMP levels underlie a daytime-dependent decrease in pheromone sensitivity and a decline in the temporal resolution of pheromone pulses.

Key words: insect olfaction, pheromone transduction, moth, cyclic nucleotide, antenna, circadian difference.

Introduction

Males of the nocturnal hawkmoth *Manduca sexta* detect the pulsatile release by female moths of the sex pheromone blend using specialized trichoid sensilla. The pheromone-sensitive sensilla are located in large numbers at the leading edge of the antenna and are each innervated by two olfactory receptor neurons (ORNs) (Sanes and Hildebrand, 1976; Keil, 1989; Lee and Strausfeld, 1990). Both ORNs are sensitive to different components of the pheromone blend. Only one of the ORNs, which elicits the larger action potentials (APs), responds to the main sex pheromone component bombykal (BAL) (Starratt et al., 1979; Tumlinson et al., 1989; Kaissling et al., 1989; Dolzer et al., 2001; Kalinová et al., 2001).

Much is already known about the peripheral signal transduction processes underlying the detection of pheromone by the male moth. Pheromone-dependent rises in inositol triphosphate (IP_3) (Breer et al., 1990) cause an influx of Ca^{2+} into insect ORNs (Stengl, 1994). This triggers the opening of Ca^{2+}-dependent ion channels involved in the generation of pheromone-dependent receptor potentials (Stengl, 1993; Stengl, 1994; Zufall and Hatt, 1991; Zufall et al., 1991). In contrast, little is known about the modulation of the pheromone transduction cascade leading to adaptation and sensitization. Previous studies in *Manduca sexta* distinguished desensitization from short-term and long-term adaptation (Dolzer et al., 2003). Desensitization is the decline in excitation, as seen during a phasic–tonic response to a stimulus of long duration, while short-term adaptation is the rapidly (within seconds to minutes) reversible reduction of sensitivity due to prior stimulation. Long-term adaptation is the more slowly occurring and longer persisting (for minutes to hours) reduction of sensitivity due to previous strong stimulation (Ziegelberger et al., 1990; Marion-Poll and Tobin, 1992; Boekhoff et al., 1993; Stengl et al., 2001; Dolzer et al., 2003). Increasing evidence supports the involvement of cyclic guanosine monophosphate (cGMP) in long-term olfactory adaptation and regulatory processes in moths. After strong pheromone stimulation, cGMP levels rise slowly in antennae

and antennal homogenates of *Antherea polyphemus* and *Bombyx mori*, remaining elevated for nearly 30 min and, thus, matching the time course of long-term adaptation (Ziegelberger et al., 1990; Boekhoff et al., 1993). In the silkmoth *Bombyx mori* the perfusion of the sensillar lymph with dibutyryl guanosine 3′,5′-cyclic monophosphate caused a suppression of the sensillar potential and a reduction of the AP response in pheromone-dependent sensilla, mimicking a state of adaptation (Redkozubov, 2000).

In tip recordings of trichoid sensilla of *Manduca sexta*, adapting pheromone stimulation shifted the dose–response curves to higher stimulus intensities (Dolzer et al., 2003). In other moths, as for *Manduca sexta*, this shift is also larger for the AP than for the sensillar potential response, suggesting the presence of more than one adaptation mechanism (Zack, 1979; Kaissling et al., 1986; Kaissling et al., 1987; Dolzer et al., 2003). At least one adaptation mechanism acts on the first step of the olfactory transduction cascade, the generation of the receptor potential, which is assumed to occur in the outer dendritic segment of the ORNs (Kaissling and Thorson, 1980; Stengl et al., 1998; Dolzer et al., 2003). An additional adaptation mechanism apparently acts on the second step of the transduction cascade, the transformation of the amplitude-modulated receptor potential into frequency-modulated APs. The APs are apparently elicited in the soma- or axon-hillock region, as indicated by the polarity of APs and the current necessary to elicit them, and not at a dendritic location, as suggested by other experiments (Kaissling and Thorson, 1980; de Kramer, 1985; de Kramer et al., 1984; Dolzer et al., 2001). The molecular basis and the behavioral consequences of these different mechanisms of insect olfactory adaptation are still not fully understood.

Previous studies have revealed that the responsiveness of male moths to pheromone is daytime-dependent. Males of the nocturnal moths *Trichoplusia ni* and *Agrotis segetum* showed a distinct daily rhythm in their response to pheromone, with a maximum response during the scotophase (Linn et al., 1996; Rosén et al., 2003). Also, daytime-dependent changes in responsiveness to pheromone persist under constant conditions so are controlled by an endogenous circadian clock (Baker and Cardé, 1979; Rosén et al., 2003). Not only male responsiveness, but also calling behavior and pheromone release of female moths, show a diurnal distribution with a maximum at the end of the scotophase for *Manduca sexta* and the middle of the scotophase for *Agrotis segetum* (Itagaki and Conner, 1988; Rosén, 2002). For *Manduca sexta* it was shown that peaks in the calling behaviour of female moths and male flight activity are correlated during the scotophase (Sasaki and Riddiford, 1984). In addition, *Spodoptera littoralis* moths mated significantly less when males and females were raised in different light:dark cycles out of phase (Silvegren et al., 2005). This indicates that circadian clocks rule rhythmic mating preference in male and female moths and that photoperiod and pheromones synchronize the mating behavior of both sexes (Silvegren et al., 2005).

To determine whether daytime-dependent changes in pheromone sensitivity already occur at the periphery in male *Manduca sexta* moths and to determine whether cGMP-dependent mechanisms of long-term adaptation might be employed, we applied the membrane-permeant cGMP analogue 8-bromo guanosine 3′,5′-cyclic monophosphate (8bcGMP) in extracellular tip recordings from trichoid sensilla at two Zeitgebertimes (ZTs; ZT 1–4 and ZT 8–11). ZT 1 is at the beginning of the day (lights on at ZT 0) when the nocturnal moths switch from their active to their inactive phase, and ZT 8 is at the middle of the day when the moths are resting. Pheromone responses to the main pheromone component BAL and spontaneous APs of unstimulated ORNs of the hawkmoth *Manduca sexta* were investigated.

Materials and methods

Animals and preparation for electrophysiological recordings

Hawkmoths *Manduca sexta* (Johannson) (Lepidoptera: Sphingidae) were raised from eggs, feeding on an artificial diet [modified after Bell and Joachim (Bell and Joachim, 1976)]. Male pupae were kept under a long-day photoperiod (17 h:7 h L:D) at 24–27°C and 40–60% relative humidity and were isolated 1 day before emergence, gently cleaned with 70% ethanol, and allowed to hatch without contact with pheromone. After their second dark phase, 30 min prior to the start of each recording, the adults were taken out of the isolation boxes and fixed in a Teflon^{TM} holder. Thus, moths at both ZTs spent the same time in the Teflon^{TM} holder. The flagellum of the right antenna was immobilized with dental wax (Boxing wax, Sybron/Kerr, Romulus, MI, USA), and the 15–16 most apical annuli were clipped off. A glass electrode filled with haemolymph Ringer (Kaissling, 1995), which served as the indifferent electrode, was inserted into the flagellar lumen. To stop the first annuli from drying out the end of the antenna was sealed with ECG electrode gel (PPG, Hellige, Freiburg, Germany) afterwards. The tips of long trichoid sensilla from the apical row on the second remaining annulus were clipped off using sharpened forceps. The recording electrode, filled with sensillum lymph Ringer (Kaissling, 1995), was slipped over one sensillum. To minimize contributions of the electroantennogram, we recorded from an annulus close to the tip of the haemolymph electrode. The connection to the amplifier inputs was established with Ag/AgCl wires immersed in the electrolytes. Signals were amplified about 200-fold in a custom-built amplifier (0 Hz–2 kHz, input impedance 10^{12} Ω), and passed through an anti-aliasing filter with a cut-off frequency of 2 kHz (900C/9L8L, Frequency Devices, Haverhill, MA, USA). For data acquisition, a Digidata 1200 B digitizer and pCLAMP software (version 8) (Axon Instruments, Union City, CA, USA) were used. The electrophysiological signal and a high-pass filtered equivalent (cut-off frequency 5 Hz) were continuously recorded on a strip chart recorder (EasyGraf, Gould, Valley View, OH, USA). Voltage polarity is given with the sensillum lymph electrode in reference to the haemolymph electrode. To be able to record in the photophase we compared the beginning of the day with the

middle of the day to search for ZT-dependent effects of 8bcGMP. All experiments were performed with room lights switched on, starting either at the beginning of the day, 1 h after lights on at ZT1, or at the middle of the day, at ZT8.

Application of 8bcGMP

Drug was applied to pheromone sensilla during tip recordings by perfusion with water-soluble agents, as first suggested by Kaissling et al. (Kaissling et al., 1991). Long-term recordings from intact animals revealed no damage of the sensilla when continued for up to several days (Dolzer et al., 2001). So we allowed the sensillum lymph ringer to passively perfuse the sensillum lymph cavity and did not apply any pressure or suction. In recordings of stimulated trichoid sensilla, 8bcGMP diluted in sensillum lymph ringer was applied over the recording electrode. Therefore, we used an altered sensillum lymph ringer with a concentration of 10 mmol l^{-1} 8bcGMP. This application method is further referred to as sensillum lymph perfusion. Biogenic amines injected into the head capsule near the base of the antenna influenced oscillations of the transepithelial potential (TEP) of trichoid sensilla (Dolzer et al., 2001). Thus, the agents were transported into the antenna after injection. In recordings of the spontaneous activity of ORNs, 8bcGMP diluted in haemolymph ringer was injected through a hole in the head capsule, which was pierced with a syringe needle approximately 1 mm dorsocaudal to the right antennal base. We injected a minimum of 3 μl of 10 mmol l^{-1} 8bcGMP solution and a maximum of 5 μl of 100 mmol l^{-1} solution, resulting in a concentration of 30–500 nmol l^{-1} 8bcGMP. Because an adult moth contains approximately 1 ml of haemolymph the final 8bcGMP concentration in the haemolymph was between 30–500 μmol l^{-1}. This application mode is further referred to as haemolymph injection. The ringers used for sensillum lymph perfusion and the respective controls were prepared with *N*-(2-hydroxyethyl)-piperazine-*N*′-(2-ethane sulfonic acid) (Hepes; all chemicals from Sigma, Deisenhofen, Germany); the ringers used for haemolymph injection were prepared with a phosphate buffer (monopotassium phosphate). The pH was adjusted to 6.5, and osmolality was adjusted to 475 mosmol l^{-1} with mannitol for sensillum lymph ringer and to 450 mosmol l^{-1} for the haemolymph ringer.

Pheromone stimulation

All recordings were performed at room temperature (18–23°C). Charcoal-filtered and moistened air was permanently blown over the preparation through a glass cartridge (13 l min^{-1}). The air stream could be redirected through cartridges containing a piece of filter paper (about 1 cm^2) loaded with synthetic bombykal (E,Z-10,12-hexadecadienal; BAL) generously provided by T. Christensen (Tucson, AZ, USA). The air stream was switched between the cartridges using solenoid valves (JFMH-5-PK and MFH-5-1/8, Festo, Esslingen, Germany) controlled by the computer. Doses of 10 μg BAL dissolved in *n*-hexane (Merck, Frankfurt, Germany) were applied to the filter papers (10 μl per paper), and the solvent was allowed to evaporate. Stimulus intensity is always given in terms of the BAL dose applied to the filter paper. The cartridges were placed in the outlet in a distance of 4.5–6 cm from the recording site about 25 s prior to stimulation. Stimuli of 10 μg BAL and 50 ms duration were applied every 5 min during a recording session of 3 h. A suction tube of 10 cm diameter was placed below the animal for rapid removal of the pheromone component after stimulation, and to avoid uncontrolled stimulation due to BAL leaking out of the stimulus cartridges. Between the recording sessions, the cartridges were stored at –20°C in individual glass scintillation vials. Control cartridges loaded with hexane alone were prepared and treated the same way. A set of stimulus cartridges was used for 5–10 recording sessions; the control cartridges were used for up to 30 recordings.

Acquisition protocols and data analysis

Each sensillum trichodeum contains two ORNs, both generating spontaneous APs and responses to different compounds of the pheromone blend. The APs of both ORNs can be distinguished by their amplitude. The spontaneous activity of the ORNs was acquired in segments of about 10 min with a sampling frequency of 19.6 kHz (Clampex 8, fixed-length events). Each AP triggered a sweep of duration 12.75 ms, and the high-pass filtered signal served as a trigger channel only. All analyses were performed using the direct-current-coupled signal. The mean voltage during the initial 2.5 ms was defined as the baseline and used to measure the TEP. The baseline of all AP sweeps was then adjusted to 0 mV to identify sweeps that were triggered by artifacts. To evaluate the shape of APs, the waveforms of both classes were averaged for intervals of 10 min. The pheromone responses were recorded in sweeps of 5161 ms duration at a continuous sampling rate of 20 kHz (Clampex 8, Episodic Stimulation Mode) with a pre-trigger portion of 180.6 ms and a post-trigger portion of 4930.2 ms. The spontaneous activity between the stimuli was recorded in segments of approximately 5 min in fixed length events, as described above. The recordings of the pheromone responses were evaluated using the Microsoft Excel Add-in XtraCell (Dolzer, 2002) and Clampfit 8. For analysis of the sensillar potential, the responses were low-pass filtered at a cut-off frequency of 50 Hz (Clampfit, Gaussian filter). The evaluated parameters of the sensillar potential (SP), as illustrated in Fig. 1A,B, were: (1) the overall amplitude (SP amplitude), (2) the initial slope between the onset of the DC response and the half-maximal SP amplitude (initial slope) and (3) the half-time of the rising phase ($t_{1/2rise}$). The second portion of the rising phase of the sensillar potential was described by an exponentional fit of first order, using only the time constant (τ). The fitting was performed with a non-iterative Chebyshev algorithm

$$f(t) = \sum_{i=1}^{n} A_i e^{-t/\tau_i} + C$$

including 4-point smoothing. For analysis of the APs (Fig. 1C), the low pass-filtered trace was subtracted from the original response. This pseudo-high-pass filtering procedure, in contrast

to actual high-pass filters, does not distort the shape of the APs, and therefore allows the analysis of their amplitude and waveform. The time and positive amplitude of every AP occurring during the 5161 ms-long sweep recorded during the stimulations was counted. When APs of both amplitude classes were observed during the response, they were analyzed separately. The AP response was characterized by: (1) the peak frequency computed from the first five interspike intervals (AP frequency; Fig. 1C); (2) the latency between the beginning of the DC response and the occurrence of the first AP (AP latency; Fig. 1B); and (3) the positive AP amplitude. The peak frequency represented the phasic part of the phasic–tonic AP

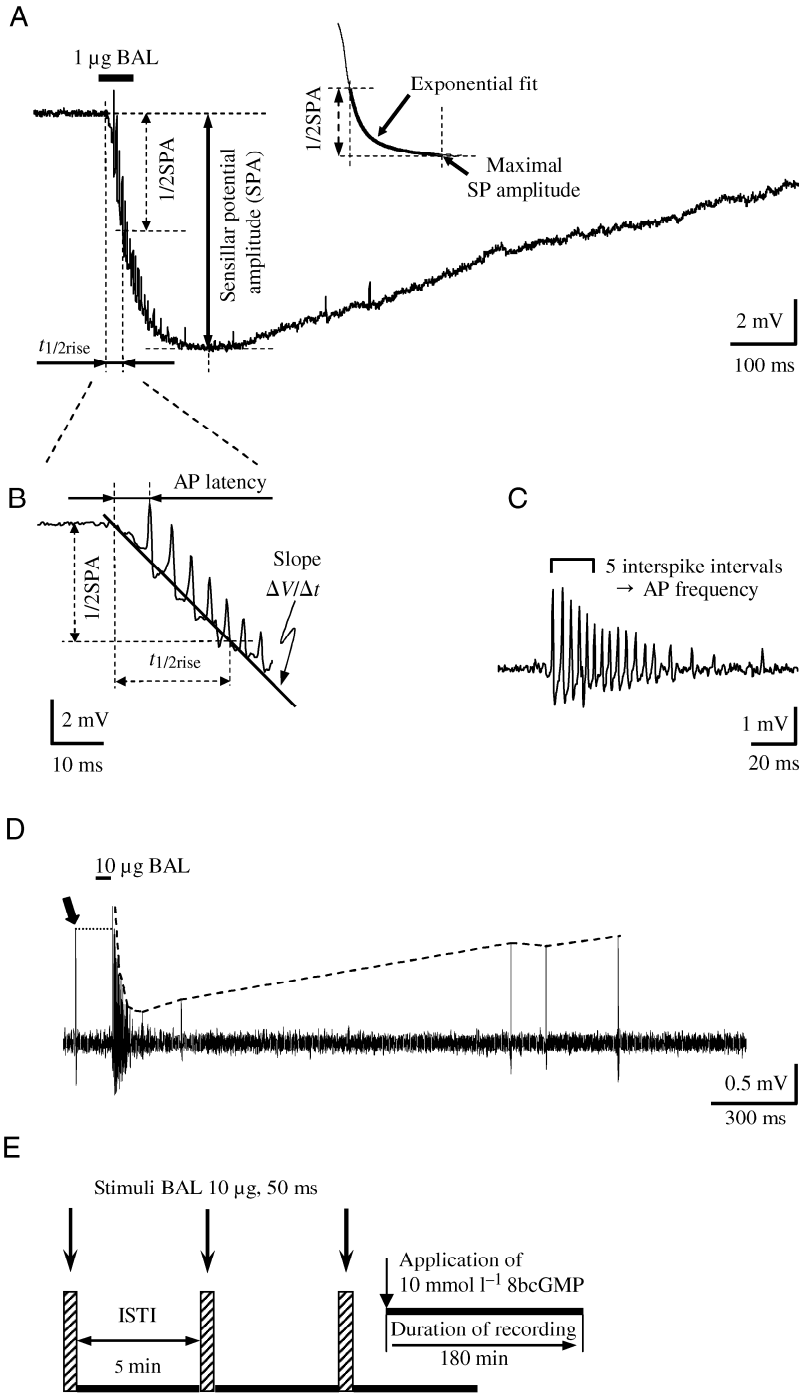

Fig. 1. The pheromone response is characterized by six parameters. (A) A non-filtered (DC) response to a 50 ms stimulus of 1 µg bombykal (BAL). Action potentials are superimposed on the negative deflection of the transepithelial potential, the sensillar potential (SP) response. The maximal SP amplitude (SPA) is measured between the baseline before the response and the negative peak of the SP. The half-time of the rising phase ($t_{1/2rise}$) is determined between the onset of the SP and the time the potential has reached 50% of the SPA (½SPA). The second portion of the rising phase is described by an exponential fit of first order, using only the time constant (τ). For the analysis of all parameters describing the SP, the responses were low-pass filtered at 50 Hz. (B) The initial phase of the response at an enlarged time scale. The initial slope is determined by dividing ½SPA by $t_{1/2rise}$. The AP latency is measured between the onset of the sensillar potential and the peak of the first action potential. (C) For the analysis of action potentials, the low-pass filtered response is subtracted from the original trace, yielding a straight baseline. The initial action potential frequency (AP frequency) is computed over the first five interspike intervals. (D) Pseudo-high-pass filtered (AC) response to a 50 ms stimulus of 10 µg BAL. The amplitudes of the large action potentials are reduced after strong BAL stimuli and regain their original amplitude in the course of several seconds (broken line). A spontaneous action potential of the non-BAL cell occurred before (filled arrow) the response. APs of the non-BAL cell can be separated from the BAL-APs by their lower and steady amplitude after stimulation with BAL. (E) Non-adapting stimulus-protocol: 50 ms long stimuli of 10 µg BAL per filter paper were applied with an interstimulus-interval (ISTI) of 5 min over a period of 180 min. 8bcGMP at 10 mmol l^{-1} was applied by perfusion over the recording electrode with the beginning of the recording [modified after (Dolzer, 2003)].

response and was calculated according to $f=N\times1000/\Sigma ISI$ [N=number of interspike intervals (ISI)]. Due to the high variability of the evaluated response parameters of each recording, the parameters were normalized to the first response of a recording. Subsequently the data for each parameter was binned to intervals of 5 min and the mean was plotted against the time of recording.

The data of the evaluated parameters of 8bcGMP and associated control recordings in each time slot were compared using the Student's t-test. For statistical analysis of changes in several parameters over the recording duration, its time course was divided into three intervals and then analyzed using a one-way ANOVA followed by the Tukey HSD *post-hoc* test. For analysis of the AP amplitude reduction, action potentials of three consecutive responses within an interval (from 1 min before to 2 min after the stimulus) were binned to 10 ms intervals and plotted against time. To measure changes in the amplitude reduction, the ratio of the minimal and maximal AP amplitudes was calculated and the normalized parameter for each recording plotted against the time of recording. To evaluate changes in the AP frequency or distribution in time, the AP response characteristics were analyzed using post-stimulus-time histograms; the AP responses were added up, binned to 10 ms intervals and plotted over time, with t=0 being the start of the DC response.

Results

In extracellular tip recordings of trichoid sensilla of the hawkmoth *Manduca sexta*, we investigated daytime-dependent effects of 8bcGMP perfusion on stimulated ORNs. The recordings were performed over the course of 3 h. Recordings started either at the beginning of the day at ZT 1, when the nocturnal moths switch from their active to their inactive phase, or at the middle of the day at ZT 8, when the moths are resting. Slow, amplitude-modulated BAL-dependent sensillar potentials with superimposed, fast, frequency-modulated APs were quantified (Fig. 1A–C). BAL stimuli were applied in a non-adapting protocol with an interstimulus interval of 5 min (Fig. 1E). In addition, the effects of 8bcGMP on spontaneous APs of unstimulated trichoid sensilla were examined.

Pheromone responses

To search for cGMP-dependent modulation of BAL responses, we evaluated various parameters of the BAL-dependent sensillar potential and AP response (Fig. 1). Parameters describing the sensillar potential are the maximum sensillar potential amplitude, the initial slope characterizing the first half, and fit τ describing the second half of the rising phase of the sensillar potential (Fig. 1A,B). Parameters describing the phasic part of the AP response are the AP frequency computed over the first five interspike intervals, the AP latency in relation to the onset of the sensillar potential, and the stimulus-dependent amplitude reduction (Fig. 1B–D). The beginning and end of the recordings were compared (Fig. 2). In both time slots from ZT 1–4 and ZT 8–11, tip recordings with perfusion of the sensillar lymph with 10 mmol l^{-1} 8bcGMP applied over the recording electrode were compared to control recordings without cyclic nucleotides. In control recordings at both ZTs, no changes in

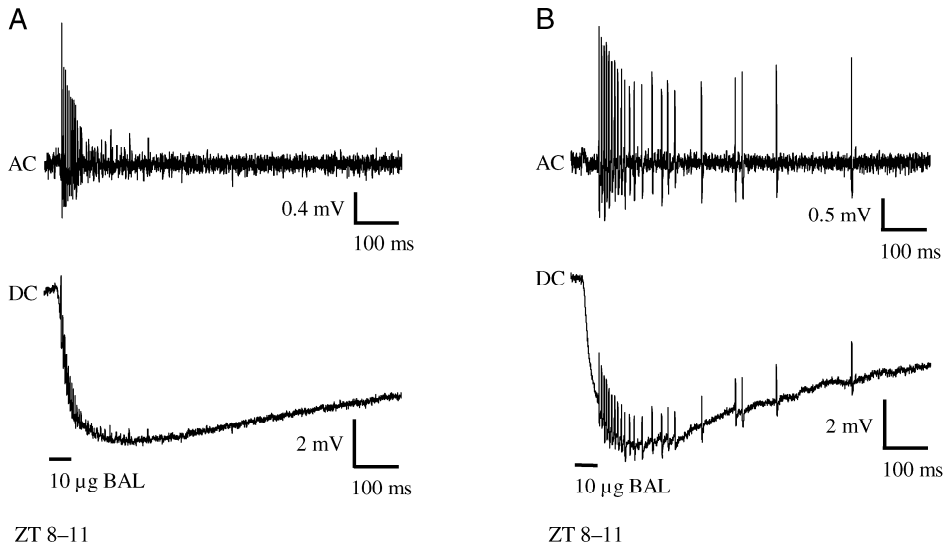

Fig. 2. Sensillum lymph perfusion with 10 mmol l^{-1} 8bcGMP at ZT 8–11 reduced the action potential (AP; top recordings) response, but left the sensillar potential (SP; bottom recordings) response unaltered. (A) At the beginning of the recording, the AP frequency in response to bombykal stimulation was 245 Hz. After 158 min (B) the SP was unaltered, but the AP frequency was reduced to 147 Hz. Also, the latency to occurrence of the first AP (AP latency) was prolonged. The effect on the AP latency was characterized by high variability between recordings. AC, pseudo-high-pass filtered signal; DC, non-filtered signal.

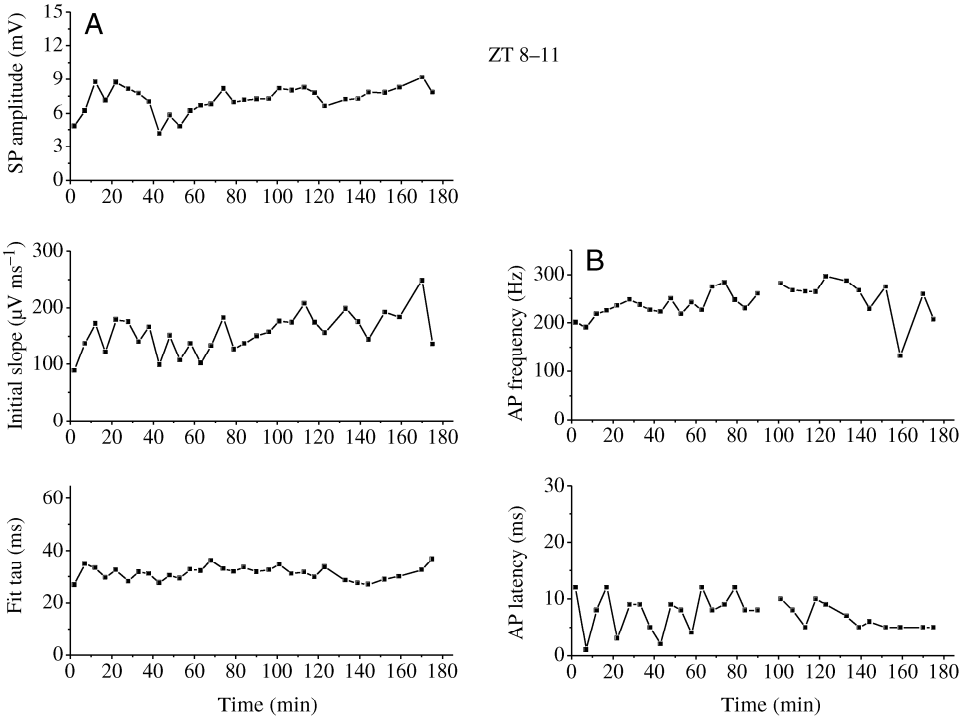

Fig. 3. Example time course of sensillar potential (A) and action potential (B) parameters for one recording under control conditions at ZT 8–11. The pheromone responses remained virtually constant during 3 h of repetitive stimulation. A bombykal stimulus of 10 μg dose and 50 ms duration was applied every 5 min.

the evaluated parameters could be observed throughout the 3 h of recordings (Figs 3, 5). In the presence of 8bcGMP the AP frequency was continuously decreased, and in a few recordings the latency between the onset of the sensillar potential and the occurrence of the first AP increased (Figs 2, 4). All other parameters that describe the AP and the sensillar potential response remained constant over the time of recording (Fig. 4). For recordings from ZT 8–11 (Fig. 5), significant differences were found in both the comparison of the AP frequency of 8bcGMP and control recordings (Student's *t*-test for independent samples, $P<0.01$) and also within the time course of the AP frequency (ANOVA and Tukey HSD *post-hoc* test; $\alpha=0.01$, $P<0.01$). No significant difference was found between intervals 2 and 3 of the time course. In contrast no significant differences were found for the sensillar potential amplitude. Due to the high variability for the AP latencies no significant difference for recordings with or without 8bcGMP was found (data not shown).

As in recordings from ZT 8–11, the AP frequency of recordings from ZT 1–4 (Fig. 5) showed a significant decrease when 10 mmol l^{-1} 8bcGMP was included in the recording electrode (Students *t*-test for independent samples, $P<0.01$), but no significant difference was found between groups 2 and 3 of the time course (ANOVA and Tukey HSD *post-hoc* test; $\alpha=0.01$, $P<0.01$). The sensillar potential amplitude and the evaluated parameters of control recordings (Fig. 5) did not change over time ($N=9$). Comparison of the AP frequency depletion between both time slots revealed a 12% stronger decrease for recordings at ZT 8–11.

It was next determined whether there are any daytime-dependent differences in the distribution of APs in response to stimulation with BAL in controls or in the presence of 8bcGMP. To analyse the distribution of APs occurring during the first 1000 ms, for responses from the beginning (0–20 min), middle (80–100 min) and end (160–180 min) of the recordings, the counted APs were binned to 10 ms intervals and plotted as post-stimulus-time histograms (Figs 6, 7). In control recordings from ZT 1–4 during the first 100 ms of the responses the response peak occurred later and the number of APs in the first 100 ms decreased significantly over the 3 h recording, leading to a slightly less phasic characteristic of the responses (Fig. 6, insert in C). Under the influence of 8bcGMP a weak decline in the number of APs over the course of the 3 h recording was also recognizable (Fig. 6A–C); owing to high variability, however, the decrease over the first 100 ms of the responses was not significant (Fig. 6C, insert).

In control recordings from ZT 8–11 the distribution of APs changed to a more tonic spike pattern (Fig. 7), which was much more pronounced than in control recordings at ZT 1–4. Also, the number of APs over the first 100 ms of the BAL

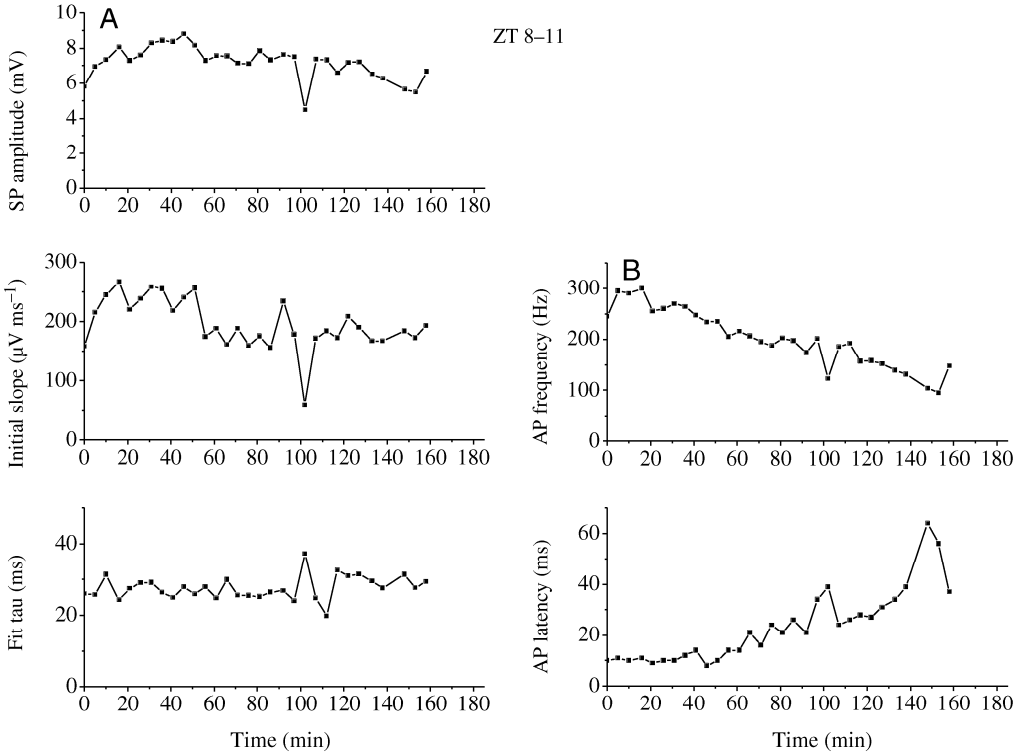

Fig. 4. Time course of sensillar potential (SP; A) and action potential (AP; B) parameters under the influence of 8bcGMP perfusion at ZT 8–11. Shown is one representative recording. During sensillum perfusion with 10 mmol l^{-1} 8bcGMP the AP frequency continuously decreased and the AP latency increased, while the parameters that describe the SP remained virtually constant.

responses significantly decreased over the course of the recordings. The numbers of APs were much more strongly decreased in control recordings at ZT 8–11 than at ZT 1–4 (Fig. 6C, Fig. 7C, inserts). In the presence of 8bcGMP the change to a more tonic spike pattern, as well as the decline in the number of APs in the first 100 ms of the BAL responses, were even more pronounced than in the controls at ZT 1–4 and at ZT 8–11.

Effects on the amplitude reduction

Characteristic for responses to BAL stimuli of higher dose is the reduction of the AP amplitude (Fig. 1C,D), which lasts for seconds to minutes. Especially in responses to strong stimuli with BAL concentrations higher than 1 μg per filter paper, the reduction of the peak-to-peak amplitude in the phasic portion is very strong and, in many cases, after about 150 ms the APs cannot be distinguished from noise. When comparing the beginning and end of a recording (Fig. 2), or the positive amplitude of APs in responses during an interval of 1 min before to 2 min after stimulation, several effects of 8bcGMP on the amplitude reduction can be observed (Fig. 8A). At the beginning of the recordings the amplitude reduction is very strong, but it gradually attenuates over the time of the recording until the reduction is only very weak and transient in the presence of 8bcGMP (Fig. 8A). In control recordings no strong changes in the amplitude reduction are recognizable (Fig. 8A). In addition to the cGMP-dependent attenuation of the decline of the AP amplitude, the kinetics of the amplitude attenuation also changed (data not shown). At the beginning of the recording the APs did not return to their prestimulus amplitude until 2 min after the stimuli had been applied. Under the influence of 8bcGMP the recovery phase was gradually attenuated until it disappeared. Also the minimal positive AP amplitude linearly increased over the course of the recording (data not shown). Furthermore, the normalized ratio of the minimal and maximal positive AP amplitude (Fig. 8B) (as a mean of the strength of the amplitude reduction) shows that 8bcGMP perfusion attenuates the AP amplitude reduction more strongly than in the controls at both ZTs. In addition, there are differences between both ZTs in the control recordings and in those with 8bcGMP perfusion. At ZT 8–11 with application of 8bcGMP, an attenuation of the amplitude reduction was recognizable in all of the recordings, whereas in the associated controls the strength of the amplitude reduction was reduced in only one recording over the entire time course of the recordings. At ZT 1–4 the recordings were more unequally distributed. In the control recordings there was higher variance compared to

recordings at ZT 8–11, which also increased with the duration of the recording. Under the influence of 8bcGMP 2 populations of recordings were recognizable. In one population that describes the majority of the recordings, the amplitude reduction increased only slightly and this increase did not start until 90 min after the beginning of the recording. In the second population a very strong attenuation of the amplitude reduction was present from the beginning. Changes in AP amplitudes of spontaneous APs between BAL responses were not found.

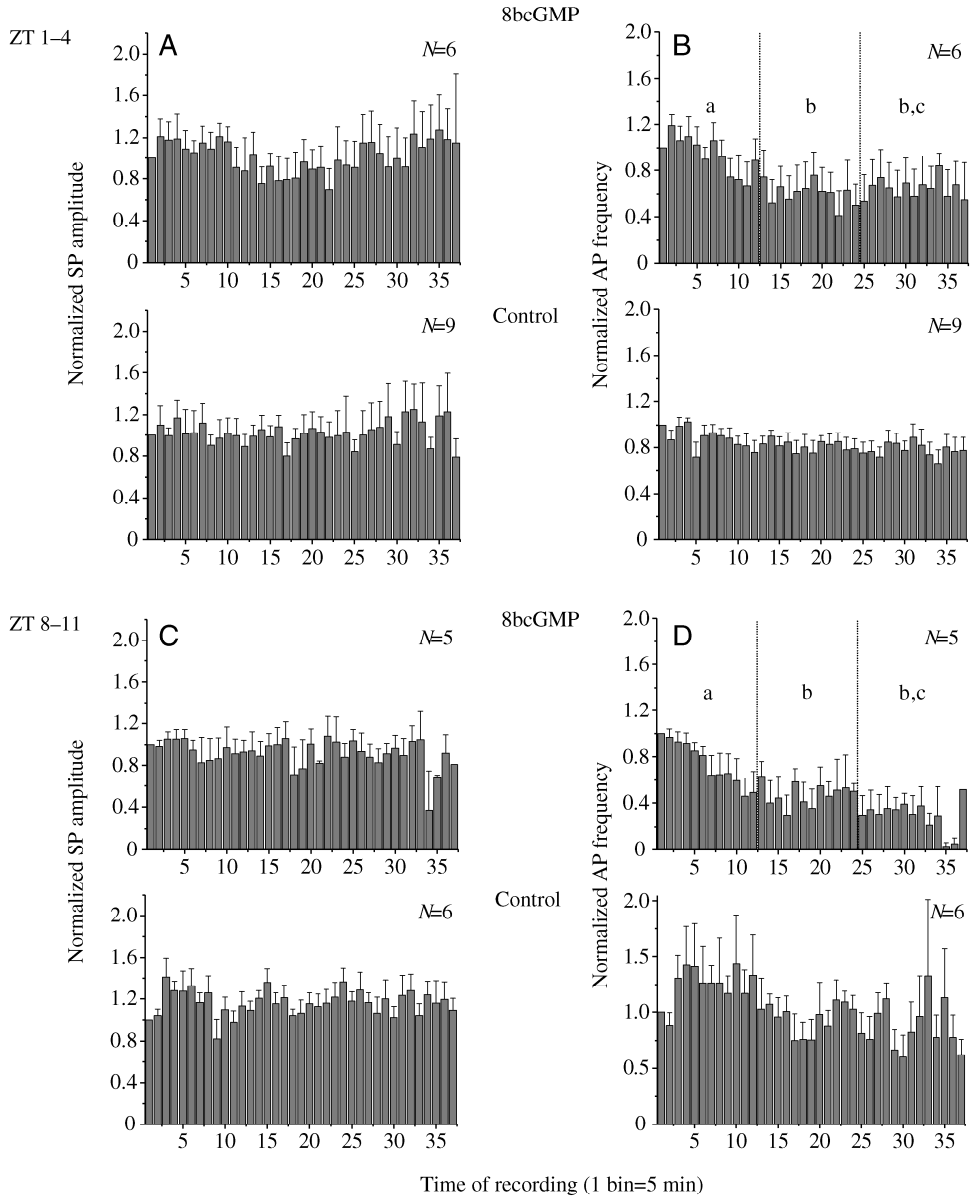

Fig. 5. The normalized and binned sensillar potential (SP) amplitude (A,C) and action potential (AP) frequency (B,D) parameters over 185 min (1 bin=5 min) for recordings from ZT 1–4 (A,B) and ZT 8–11 (C,D). Values are means + s.c.m. (B,D) Under the influence of 8bcGMP (top recordings) a significant decrease of the AP frequency can be recognized for both ZTs when compared to the respective controls (bottom recordings) (Student's *t*-test for independent samples, $P<0.01$) and when comparing the three intervals within each time course (separated by dotted lines). Different lower case letters denote significant differences between tested groups of means (ANOVA and Tukey HSD *post-hoc* test; $\alpha=0.01$, $P<0.01$). The means in the second (b) and third time interval (c) are not significantly different (b,c) for both ZTs. The decrease of the action potential frequency at ZT8–11 is 12% stronger than in recordings from ZT1–4. (A,C) In contrast, the SP amplitude remained unchanged.

Spontaneous APs

In addition to the effects of 8bcGMP on pheromone responses, 8bcGMP also had an effect on the waveform of spontaneous APs of unstimulated sensilla trichoidea of isolated male moths that had never experienced pheromone. Changes in the waveform of both AP classes were observed after 6 of 8 haemolymph injections of 30–500 nmol 8bcGMP for several hours. In one recording (Fig. 9) the time course of the AP waveform could be monitored almost continuously for 6 h after the injection at the base of the antenna. The waveforms of both AP classes were influenced independently, suggesting that the 8bcGMP effect took place within the individual ORNs (N=6). After injection of 8bcGMP, two aspects of the waveform were altered: the peak-to-peak amplitude increased and the negative phase of the APs was prolonged. These effects then reverted and reappeared later, suggesting the presence of feedback-coupled mechanisms that were triggered by 8bcGMP injection. A consistent change in the frequency or burst behaviour of the APs was not observed. In recordings of stimulated olfactory neurons no effects of 8bcGMP on the waveform of spontaneous APs between the stimuli were found (data not shown).

Discussion

Using long-term tip recordings from pheromone-sensitive trichoid sensilla of the hawkmoth *Manduca sexta*, we tested whether cGMP affects responses to stimulation with the main pheromone component BAL in a daytime-dependent manner. Perfusion of the sensillum lymph with 10 mmol l^{-1} of the membrane-permeable cGMP analogue 8bcGMP reduced the AP frequency of ORNs in response to BAL stimulation in a daytime-dependent manner. The reduction was 12% stronger at the middle of the day (ZT 8–11) than in recordings starting at ZT 1. Also, in control recordings daytime-dependent differences in the AP distribution of the pheromone responses were observed. Pheromone responses at ZT 8–11 became increasingly tonic and fewer APs were elicited within the first 100 ms of the responses. At ZT 8–11 only, 8bcGMP also strongly decreased the number of APs occurring during the first 100 ms of the response, leading to a more tonic AP response. The 8bcGMP perfusion also attenuated the reduction of the AP amplitude in most of the recordings from ZT 8–11 and in a subpopulation of recordings from ZT 1–4. Furthermore, the injection of 8bcGMP into the haemolymph altered the

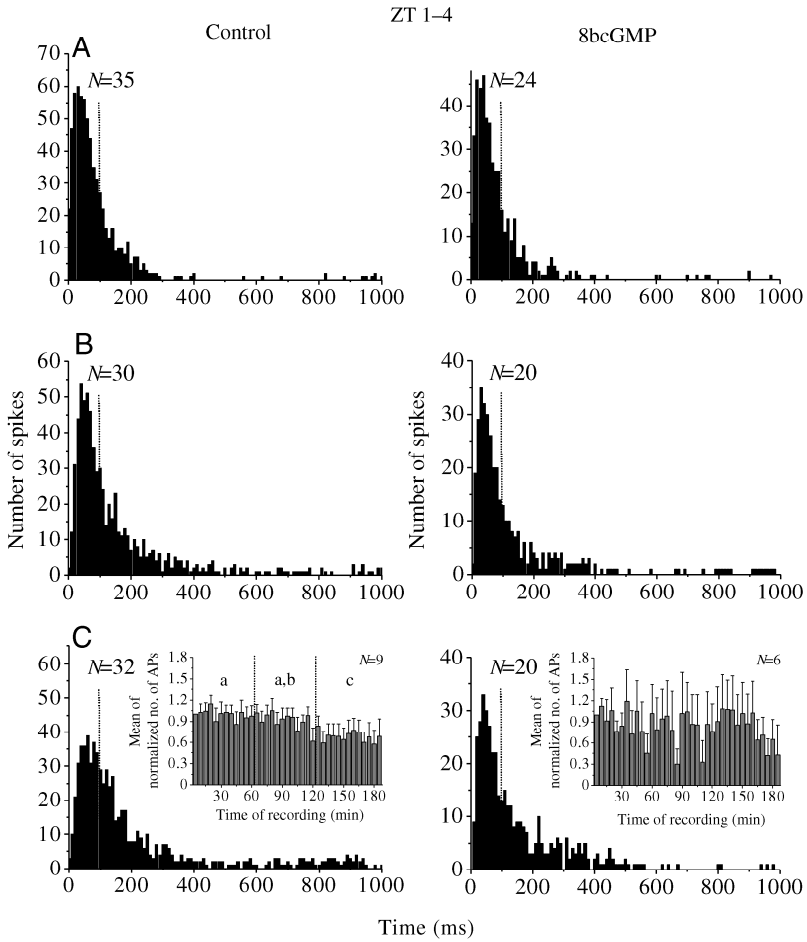

Fig. 6. Action potential (AP) distribution in responses to stimulation with 10 μg bombykal. Post-stimulus-time histograms (binwidth= 10 ms) for recordings from ZT 1–4 for the beginning (A; 0–20 min), middle (B; 80–100 min), and end of the recordings (C; 160–180 min). In the control recordings (left) the numbers of APs in the first part of the phasic response decreased. Also, the number of APs over the first 100 ms of the responses (insert in C) showed a slight but significant decline at the end of the recording duration. Under the influence of 8bcGMP (right) no changes are recognizable. Values in inserts are means + s.e.m. Since the sample sizes differed for the different time windows all y-axes were scaled to $y=n\times2$ (dotted line=100 ms after the onset of the sensillar potential). Different lower case letters denote significant differences between tested groups of means (separated by dotted lines in the insert) (ANOVA and Tukey HSD *post-hoc* test; α=0.01, P<0.01).

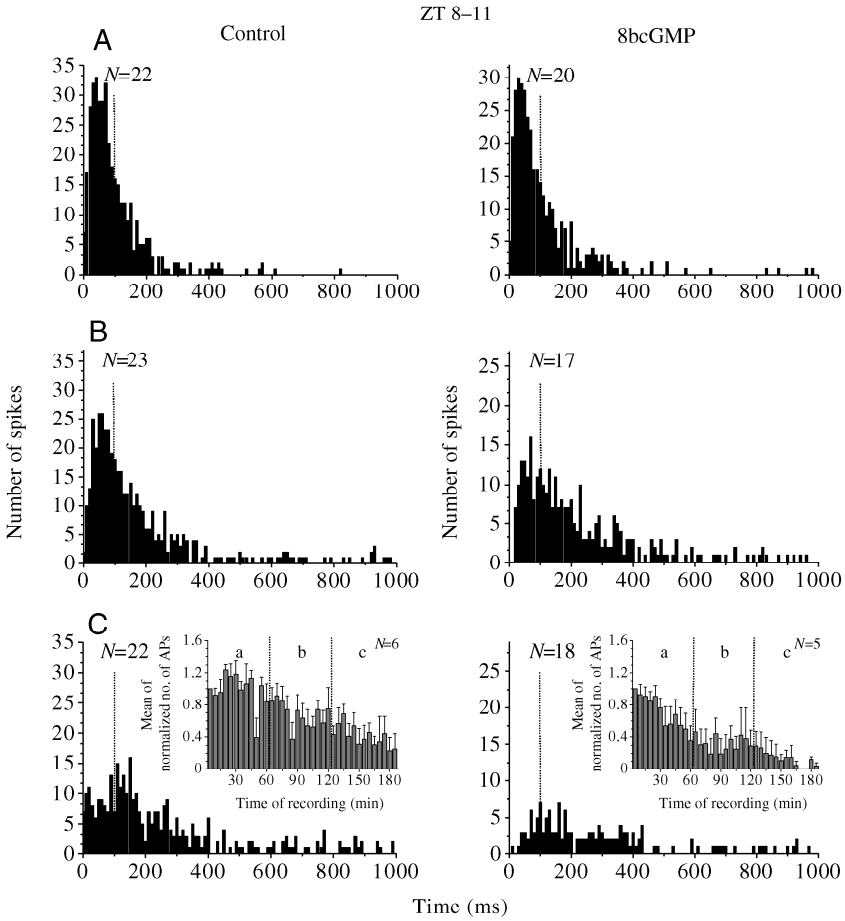

Fig. 7. Action potential (AP) distribution in responses to stimulation with 10 μg bombykal. Post-stimulus-time histograms (binwidth=10 ms) for recordings from ZT 8–11 for the beginning (A; 0–20 min), middle (B; 80–100 min) and end of the recordings (C; 160–180 min). Both in the controls (left) as well as under the influence of 8bcGMP (right) the first phasic part of the AP response declined, leading to a more tonic response. Also, the numbers of APs over the first 100 ms (insert in C) showed a significant decrease both with 8bcGMP and in the controls. This decline was stronger in the presence of 8bcGMP. Since the sample sizes differed for the different time windows all *y*-axes were scaled to $y=n\times1.6$ (dotted line=100 ms after the onset of the sensillar potential). Different lower case letters in the inserts denote significant differences between tested groups of means, which are separated by dotted lines (ANOVA and Tukey HSD post-hoc-test; $\alpha=0.01$, $P<0.01$).

waveform of spontaneous APs of unstimulated ORNs. The sensillar potential in contrast remained unaffected at all ZTs.

The role of cGMP in insect olfactory adaptation

It was known from extracellular tip recordings from different moth species that strong, long or high frequency pheromone stimuli can cause desensitization, short- or long-term adaptation to pheromone responses depending on stimulus strength and time course (Zack, 1979; Dolzer et al., 2003; Marion-Poll and Tobin, 1992). In *Manduca sexta* it was shown that, depending on BAL stimulus, length and strength parameters of the sensillar potential and AP response adapt differentially (Dolzer et al., 2003). After adapting to pheromone stimuli, the time of rise of the sensillar potential did not increase after short-, only after long-adapting stimuli, whereas both stimulation schemes shifted the dose–response curves of the sensillar potential amplitude, as well as the initial slope of its rising phase, to higher stimulus concentrations. The shift in the dose–response curve of the AP response was larger compared to the shift in dose–response curve of the sensillar potential response, indicating that depending on the properties of the stimulus unknown mechanisms of adaptation occur at different levels of the transduction process (Dolzer et al., 2003).

Biochemical experiments implicated that rises in cGMP concentration are involved in at least one mechanism of long-term adaptation, because strong and long pheromone stimuli caused delayed and sustained rises in intracellular cGMP concentrations in antennal homogenates of *Antherea polyphemus* and *Bombyx mori*. These increased levels of cGMP concentration matched the time courses of long-term adaptations (Ziegelberger et al., 1990; Boekhoff et al., 1993). In immunocytochemical experiments and *in situ* hybridizations it could be shown that at least subpopulations of pheromone-sensitive ORNs of male *Manduca sexta* upregulate cGMP concentrations after minute-long (but not seconds-long) exposure to female pheromones (Stengl et al., 2001). These cGMP rises were augmented *via* exposure to NO donors in the presence of a pheromone-inducible NO-synthase-like enzyme in ORNs, but not *via* stimulation of a soluble guanylyl cyclase (Stengl and Zintl, 1996; Stengl et al., 2001). Thus, adapting, long and strong pheromone stimuli, as occur in close vicinity to the calling female, cause long-lasting rises in cGMP concentrations. But it remained unknown which antennal

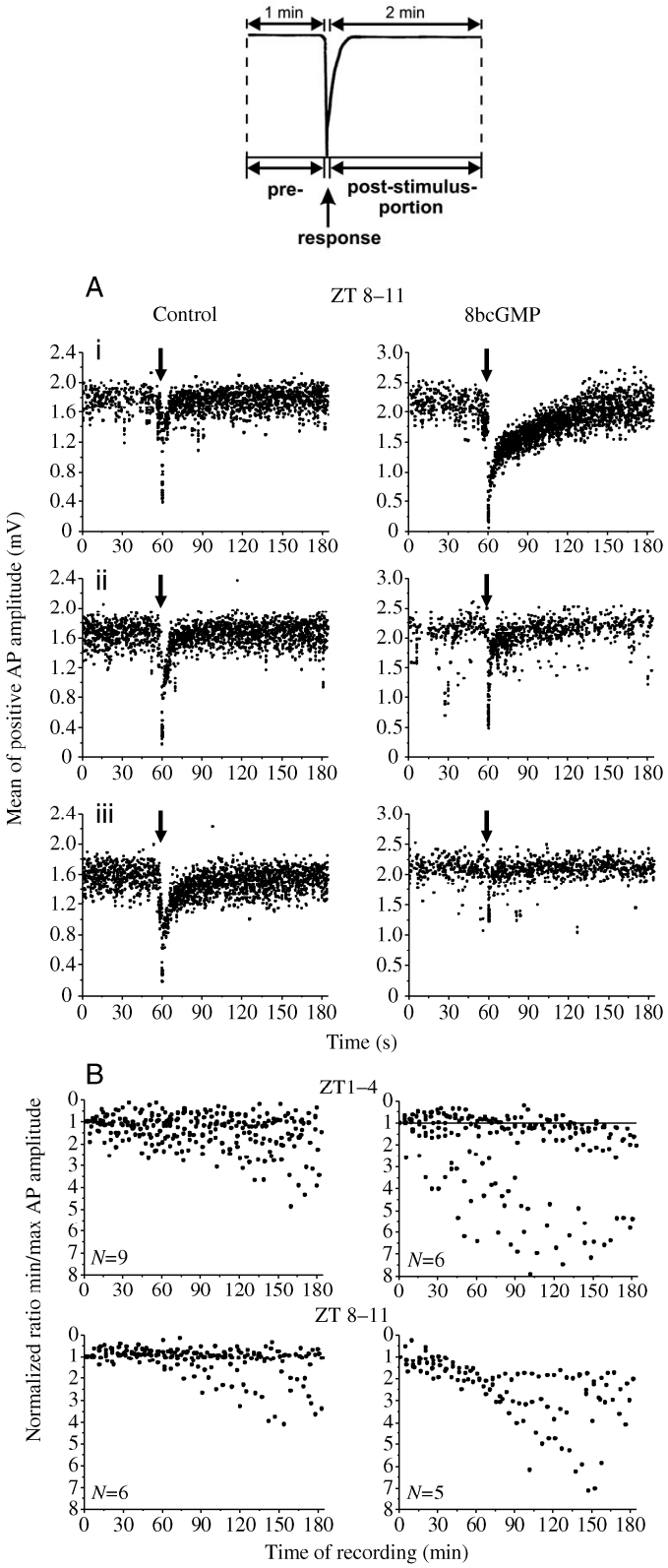

Fig. 8. Analysis of the amplitude reduction of action potentials (APs). (A) The 8bcGMP-dependent AP amplitude reduction for the beginning (i), the middle (ii) and the end (iii) of one recording at ZT 8–11. Plots show the mean of the positive amplitude of APs that occurred during an interval of 1 min before to 2 min after the stimulation. Arrows indicate the time of stimulation. Each plot consists of the binned (binwidth=10 ms) and averaged AP amplitudes of three consecutive responses to a stimulus of 10 μg BAL. For the first three responses of the recording the AP amplitudes were strongly reduced. The APs returned to its pre-stimulus amplitude not until about 2 min after the stimuli were applied. After about 90 min the slow portion of the recovering phase disappeared. Also, the amplitude reduction showed a slight decrease. At the end of the recording a strong 8bcGMP-dependent decrease in the amplitude reduction was observed. The amplitude reduction was only weak and transient. In the control recordings no or only weak fluctuations in the reduction of positive APs were found. In contrast to the recording with 8bcGMP the slowly recovering phase is even more prominent at the end of the recording. (Due to the non-linear change of the BAL-AP amplitude in the post-stimulus portion BAL- and non-BAL APs could not be distinguished). (B) Normalized ratio between the minimal and maximal positive AP amplitude of a response as a mean of the strength of the amplitude reduction. Each recording was normalized to the first value. Values >1 represent a decrease in the reduction of the AP amplitude. In recordings from ZT 1–4 with 10 mmol l^{-1} 8bcGMP diluted in the sensillum lymph ringer (top right), two populations of recordings can be observed. One population showed a very strong increase from the beginning on, the other one resembled the time course of the reduction in control recordings. For control recordings from ZT 1–4 (top left), most of the data points showed a cumulative composition at the beginning followed by an increasing variance leading to a continuous broadening in the distribution later in the recordings. Under the influence of 8bcGMP all of the recordings from ZT 8–11 (bottom right) showed an increase, whereas in the associated controls (bottom left) most data points were located in a relatively distinct band around 1.

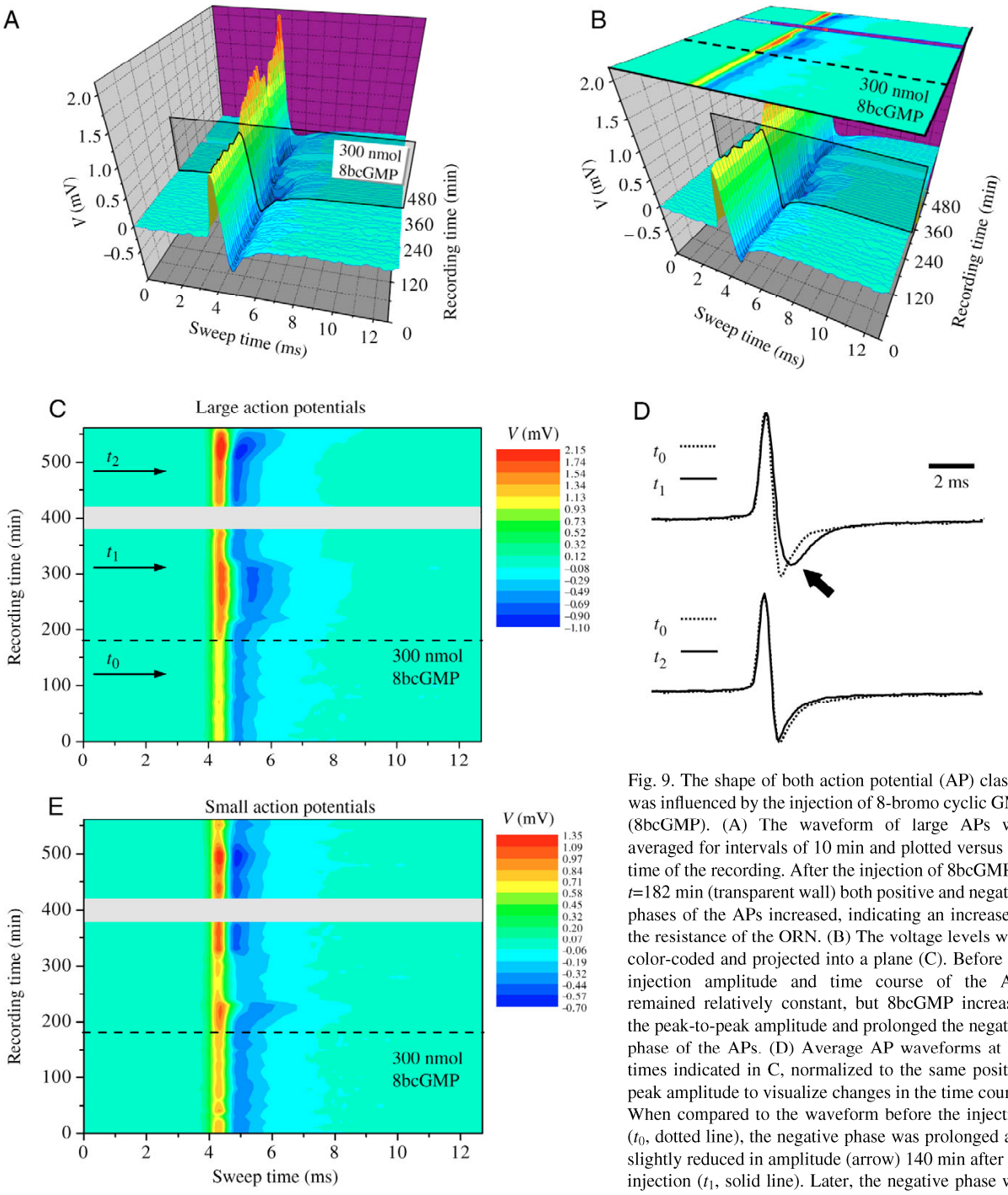

Fig. 9. The shape of both action potential (AP) classes was influenced by the injection of 8-bromo cyclic GMP (8bcGMP). (A) The waveform of large APs was averaged for intervals of 10 min and plotted versus the time of the recording. After the injection of 8bcGMP at t=182 min (transparent wall) both positive and negative phases of the APs increased, indicating an increase in the resistance of the ORN. (B) The voltage levels were color-coded and projected into a plane (C). Before the injection amplitude and time course of the APs remained relatively constant, but 8bcGMP increased the peak-to-peak amplitude and prolonged the negative phase of the APs. (D) Average AP waveforms at the times indicated in C, normalized to the same positive peak amplitude to visualize changes in the time course. When compared to the waveform before the injection (t_0, dotted line), the negative phase was prolonged and slightly reduced in amplitude (arrow) 140 min after the injection (t_1, solid line). Later, the negative phase was shortened again, and 300 min after the injection the time course of the averaged and normalized waveform (t_2, solid line) was identical to the pre-injection waveform (t_0, dotted line). (E) Changes in the waveform of the small APs also showed increases of the peak-to-peak amplitude and a prolongation of the negative phase, but over a different time course. The prolongation of the negative phase reversed after 50 min, while with the large APs the reversal occurred after 150 min. The peak-to-peak amplitude of the small APs was transiently reduced back to the pre-injection level between 70 and 120 min after the injection, while the large APs reached their highest peak-to-peak amplitude at the same time. During the gap in the data (gray areas in C and E), small and large APs could not be distinguished.

targets are affected *via* cGMP and whether rises in cGMP concentration underlie at least one mechanism of long-term olfactory adaptation.

In the present study we have shown in tip recordings of trichoid sensilla of the hawkmoth *Manduca sexta* that brief but strong pheromone stimuli applied in a non-adapting protocol with interstimulus intervals of 5 min cause no changes in the pheromone response, either in the sensillar potential or in the AP response. Only during long-term exposure to the cGMP analogue 8bcGMP was the AP frequency decreased over the 3 h of the recordings; the sensillar potential response remained unchanged. This selective decrease in the AP frequency distinguishes one of the different forms of adaptation in *Manduca sexta* for the first time. Interestingly, injection of dibutyryl guanosine 3′,5′-cyclic monophosphate into the antennae of *Bombyx mori* decreased the AP frequency as well as the sensillar potential amplitude (Redkozubov, 2000). The recording methods and the cGMP analogues employed differed between both moth species, as well as the stimulation scheme. While only very brief strong pheromone stimuli were applied in our recordings, Redkozubov employed either 10 times longer and stronger pheromone stimuli, or presented continuous pheromone stimuli of a lower dose. Most likely, these differing pheromone stimulation protocols were responsible for the differences observed.

The decrease in the AP frequency, the decrease in the number of APs in the first 100 ms of the response, and the observed cGMP-dependent slowdown of the negative phase of spontaneous APs, could all be explained by a closure of K^+-channels. Also, the increase in the peak-to-peak amplitude of spontaneous APs could be explained by the closure of K^+-channels resulting in an increased resistance. This increase in the resistance of the preparation could also be responsible for the 8bcGMP-dependent attenuation of the AP amplitude reduction. In *Manduca sexta*, a pheromone-activated, cGMP-blockable K^+-channel was described without its function being understood (Zufall et al., 1991; Stengl et al., 1992). Based on our findings and on FURA-measurements with cultured ORNs from *Manduca sexta* (M.S., unpublished observations), we assume that strong or long pheromone stimulation resulted in long-term rises of intracellular Ca^{2+} levels. These Ca^{2+} elevations then appeared to cause rises of intracellular cGMP levels, which then close the fast BAL-dependent cGMP-dependent K^+-channel. Whether other cGMP-gated ion channels that were described in patch clamp recordings in *Manduca sexta* (Dolzer, 2002) are also involved in the observed adaptations of the action potential response remains to be shown.

Daytime-dependent differences in the cGMP effects

In addition to daytime-dependent differences in the control recordings, several daytime-dependent effects of cGMP were found. 8bcGMP-dependent distribution of APs became more tonic and the number of APs during the first 100 ms of the BAL response only decreased at ZT 8–11. Furthermore, the 8bcGMP-dependent decrease of the AP frequency at ZT 8–11 was 12% stronger than in recordings at ZT 1–4. Also, the 8bcGMP-dependent attenuation of the reduction of the positive AP amplitude was more prominent at ZT 8–11. In addition to daytime-dependent differences in the effects of 8bcGMP, a different distribution in the timing of APs in control recordings between both ZTs were observed. In recordings from ZT 8–11 the pheromone-dependent AP response became less phasic and more tonic, and also fewer numbers of APs were elicited during the first 100 ms of the BAL response, than at ZT 1–4. Thus, the main differences between the responses of the recordings at ZT 1–4 and ZT 8–11 are the distribution and numbers of APs elicited in the phasic portion of the response. An ORN with more tonic responses would very likely be less able to resolve pheromone filaments of high frequencies.

The effects of the plume structure on behavioural and flight responses of male *Cadra cautella* to pheromone were investigated in wind tunnel experiments (Mafra-Neto and Cardé, 1995a; Mafra-Neto and Cardé, 1995b). Faster frequencies of pulses were followed by a higher percentage of males responding by shorter latencies and less time spent in the search behaviour. Because the variation of the fine structure of the plume had more influence on the flight pattern of males than a 1000-fold increase in the pheromone dosage, the temporal resolution of ORNs appears to be very crucial. In addition, it was shown that the antennal lobe network is strongly modulated by the temporal pattern of the stimulus (Christensen et al., 1998a; Christensen et al., 1998b). The antennal lobe is tuned to fast temporal discrimination of pheromone pulses, which appears to be necessary for odor blend discrimination (Christensen and Hildebrand, 1997; Stopfer et al., 1997). Whether the decrease of the numbers of APs within the first 100 ms represents a further adaptation mechanism that decreases the ability of the ORNs to encode pulsed pheromone signals and therefore blend discrimination remains to be shown in future investigations.

Our results suggest for the first time that there might be daytime-dependent differences in the sensitivity of moth ORNs to pheromone. Whether the observed differences are controlled by an endogenous circadian clock that regulates the sensitivity of ORNs remains to be studied. So far, it has been assumed that daytime-dependent rhythms in male responsiveness to pheromone occur at the level of the antennal lobe and midbrain (Payne et al., 1969; Worster and Seabrook, 1988; Rosén et al., 2003). In addition, investigations of circadian sensitivity changes to food odours in *Leucophaea maderae* indicated that these rhythms were driven by a circadian pacemaker in the optic lobes but not in the ORNs (Page and Koelling, 2003), while circadian changes in the electroantennogram (EAG) in *Drosophila melanogaster* suggested that they depend on PER-dependent endogenous circadian pacemakers within the antenna (Krishnan et al., 1999; Tanoue et al., 2004; Zhou et al., 2005). The main difference between our experiments and the studies mentioned is that we performed tip- instead of EAG-recordings, which allowed us to analyse the AP response of a single ORN with high temporal and spatial resolution. The shift from a phasic-tonic to a tonic response pattern as an effect of 8bcGMP application and circadian differences has never been

shown before because it cannot be resolved by the EAG-recording technique.

It is still unknown whether *Manduca sexta* males are maximally sensitive to their intermittently pulsed pheromone signals during the late night when the females are calling. Also, it is unresolved whether there is a circadian rhythm that might adapt the ORNs in the photophase when no pheromone is released by the females and when the moths are inactive. To investigate rhythms in the sensitivity of ORNs to pheromone and to challenge the hypothesis that the blend discrimination is affected *via* a cGMP-dependent mechanism, long-term recordings starting at the late scotophase, which comprises the shift from scoto- to photophase, will be performed. In addition, it will be investigated in behavioral studies whether male *Manduca sexta* show rhythmic changes in their responsiveness to pheromone. Furthermore, current biochemical experiments examine whether there are differences in cyclic nucleotide concentrations in the moth antenna at different ZTs depending on differing stimulation schemes (K. Riedinger and M.S., unpublished).

List of abbreviations

AP	action potential
BAL	bombykal
cGMP	cyclic guanosine monophosphate
EAG	electroantennogram
IP_3	inositol triphosphate
ISI	interspike interval
ORN	olfactory receptor neuron
SP	sensillar potential
SPA	SP amplitude
TEP	transepithelial potential
ZT	Zeitgebertime

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References

Baker, T. C. and Cardé, R. T. (1979). Endogenous and exogenous factors affecting periodicities of female calling and male sex pheromone response in *Grapholitha molesta* (Busck). *J. Insect Physiol.* **25**, 943-950.

Bell, R. A. and Joachim, F. A. (1976). Techniques for rearing laboratory colonies of tobacco hornworms and pink bollworms. *Ann. Ent. Soc. Am.* **69**, 365-373.

Boekhoff, I., Seifert, E., Göggerle, S., Lindemann, M., Krüger, B. W. and Breer, H. (1993). Pheromone-induced second-messenger signaling in insect antennae. *Insect Biochem. Mol. Biol.* **23**, 757-762.

Breer, H., Boekhoff, I. and Tareilus, E. (1990). Rapid kinetics of second messenger formation in olfactory transduction. *Nature* **395**, 65-68.

Christensen, T. A. and Hildebrand, J. G. (1997). Coincident stimulation with pheromone components improves temporal pattern resolution in central olfactory neurons. *J. Neurophysiol.* **77**, 775-781.

Christensen, T. A., Waldrop, B. R. and Hildebrand, J. G. (1998a). GABAergic mechanisms that shape the temporal response to odors in moth olfactory projection neurons. *Ann. NY Acad. Sci.* **855**, 475-481.

Christensen, T. A., Waldrop, B. R. and Hildebrand, J. G. (1998b). Multitasking in the olfactory system: context-dependent responses to odors reveal dual GABA-regulated coding mechanisms in single olfactory projection neurons. *J. Neurosci.* **18**, 5999-6008.

de Kramer, J. J. (1985). The electrical circuitry of an olfactory sensillum in *Antheraea polyphemus*. *J. Neurosci.* **5**, 2484-2493.

de Kramer, J. J., Kaissling, K.-E. and Keil, T. A. (1984). Passive electrical properties of insect olfactory sensilla may produce the biphasic shape of spikes. *Chem. Senses* **8**, 289-295.

Dolzer, J. (2002). Mechanisms of modulation and adaptation in pheromone-sensitive trichoid sensilla of the hawkmoth *Manduca sexta.* PhD thesis, Philipps-Universität, Marburg, Germany. http://archiv.ub.uni-marburg.de/diss/z2002/0185.

Dolzer, J., Krannich, S., Fischer, K. and Stengl, M. (2001). Oscillations of the transepithelial potential of moth olfactory sensilla are influenced by octopamine and serotonin. *J. Exp. Biol.* **204**, 2781-2794.

Dolzer, J., Fischer, K. and Stengl, M. (2003). Adaptation in pheromone-sensitive trichoid sensilla of the hawkmoth *Manduca sexta*. *J. Exp. Biol.* **206**, 1575-1588.

Itagaki, H. and Connor, W. E. (1988). Calling behavior of *Manduca sexta* (L.) (Lepidoptera: Sphingidae) with notes on the morphology of the female sex pheromone gland. *Ann. Entomol. Soc. Am.* **81**, 798-807.

Kaissling, K.-E. (1995). Single unit and electroantennogram recordings in insect olfactory organs. In *Experimental Cell Biology of Taste and Olfaction* (ed. A. I. Spielmann and J. G. Brand), pp. 361-377. Boca Raton, NY: CRC Press.

Kaissling, K.-E. and Thorson, J. (1980). Insect olfactory sensilla: structural, chemical and electrical aspects of the functional organization. In *Receptors for Neurotransmitters, Hormones and Pheromones in Insects* (ed. D. B. Satelle, L. M. Hall and J. G. Hildebrand), pp. 261-282. Amsterdam: Elsevier, North-Holland.

Kaissling, K.-E., Zack-Strausfeld, C. and Rumbo, E. R. (1986). Adaptation processes in insect olfactory receptors: their relation to transduction and orientation. *Chem. Senses* **11**, 574.

Kaissling, K.-E., Zack-Strausfeld, C. and Rumbo, E. R. (1987). Adaptation processes in insect olfactory receptors. Mechanisms and behavioral significance. *Ann. NY Acad. Sci.* **510**, 104-112.

Kaissling, K.-E., Hildebrand, J. G. and Tumlinson, J. H. (1989). Pheromone receptor cells in the male moth *Manduca sexta*. *Arch. Insect Biochem. Physiol.* **10**, 273-279.

Kaissling, K.-E., Keil, T. A. and Williams, J. L. D. (1991). Pheromone stimulation in perfused sensory hairs of the moth *Antheraea polyphemus*. *J. Insect Physiol.* **37**, 71-78.

Kalinová, B., Hoskovec, M., Liblikas, I., Unelius, C. R. and Hansson, B. S. (2001). Detection of sex pheromone components in *Manduca sexta* (L.). *Chem. Senses* **26**, 1175-1186.

Keil, T. A. (1989). Fine structure of the pheromone-sensitive sensilla on the antenna of the hawkmoth, *Manduca sexta*. *Tissue Cell* **21**, 139-151.

Krishnan, B., Dryer, S. E. and Hardin, P. E. (1999). Circadian rhythms in olfactory responses of *Drosophila melanogaster*. *Nature* **400**, 375-378.

Lee, J. K. and Strausfeld, N. J. (1990). Structure, distribution, and number of surface sensilla and their receptor cells on the antennal flagellum of the male sphinx moth *Manduca sexta*. *J. Neurocytol.* **19**, 519-538.

Linn, C. E., Campbell, M. G., Poole, K. R., Wu, W.-Q. and Roelofs, W. L. (1996). Effects of photoperiod on the circadian timing of pheromone response in male *Trichoplusia ni*. *J. Insect Physiol.* **42**, 881-891.

Mafra-Neto, A. and Cardé, R. T. (1995a). Influence of plume structure and pheromone concentration on upwind flight of *Cadra cautella* males. *Physiol. Entomol.* **20**, 117-133.

Mafra-Neto, A. and Cardé, R. T. (1995b). Effect of the fine-scale structure of pheromone plumes: pulse frequency modulates activation and upwind flight of almond moth males. *Physiol. Entomol.* **20**, 229-242.

Marion-Poll, F. and Tobin, T. R. (1992). Temporal coding of pheromone pulses and trains in *Manduca sexta*. *J. Comp. Physiol A* **171**, 505-512.

Page, T. L. and Koelling, E. (2003). Circadian rhythm in olfactory response in the antennae controlled by the optic lobe in the cockroach. *J. Insect Physiol.* **49**, 697-707.

Payne, T. L., Shorey, H. H. and Gaston, L. K. (1969). Sex pheromones of noctuid moths: Factors influencing antennal responsiveness in males of *Trichoplusia ni*. *J. Insect Physiol.* **16**, 1043-1055.

Redkozubov, A. (2000). Guanosine 3′,5′-cyclic monophosphate reduces the response of the Moth's olfactory receptor neuron to pheromone. *Chem. Senses* **25**, 381-385.

Rosén, W. Q. (2002). Endogenous control of circadian rhythms of pheromone production in the turnip moth *Agrotis segetum*. *Arch. Insect Biochem. Physiol.* **50**, 21-30.

Rosén, W. Q., Han, G. B. and Löfstedt, C. (2003). The circadian rhythm of the sex-pheromone-mediated behavioral response in the turnip moth, *Agrotis segetum*, is not controlled at the peripheral level. *J. Biol. Rhythms* **18**, 402-408.

Sanes, J. R. and Hildebrand, J. G. (1976). Origin and morphogenesis of sensory neurons in an insect antenna. *Dev. Biol.* **51**, 300-319.

Sasaki, M. and Riddiford, L. M. (1984). Regulation of reproductive behaviour and egg maturation in the tobacco hawk moth, *Manduca sexta*. *Physiol. Entomol.* **9**, 315-327.

Silvegren, G., Löfstedt, C. and Rosén, W. Q. (2005). Circadian mating activity and effect of pheromone pre-exposure on pheromone response rhythms in the moth *Spodoptera littoralis*. *J. Insect Physiol.* **51**, 277-286.

Starratt, A. N., Dahm, K. H., Allen, N., Hildebrand, J. G., Payne, T. L. and Röller, H. (1979). Bombykal, a sex pheromone of the sphinx moth *Manduca sexta*. *Z. Naturforsch.* **34**, 9-12.

Stengl, M. (1993). Intracellular-messenger-mediated cation channels in cultured olfactory receptor neurons. *J. Exp. Biol.* **178**, 125-147.

Stengl, M. (1994). Inositol-trisphosphate-dependent calcium currents precede cation currents in insect olfactory receptor neurons in vitro. *J. Comp. Physiol. A* **174**, 187-194.

Stengl, M. and Zintl, R. (1996). NADPH diaphorase activity in the antennae of the hawkmoth *Manduca sexta*. *J. Exp. Biol.* **199**, 1063-1072.

Stengl, M., Zufall, F., Hatt, H. and Hildebrand, J. G. (1992). Olfactory receptor neurons from antennae of developing male *Manduca sexta* respond to components of the species-specific sex pheromone *in vitro*. *J. Neurosci.* **12**, 2523-2531.

Stengl, M., Ziegelberger, G., Boekhoff, I. and Krieger, J. (1998). Perireceptor events and transduction mechanisms in insect olfaction. In *Insect Olfaction* (ed. B. S. Hansson), pp. 49-66. Berlin, New York, Heidelberg: Springer.

Stengl, M., Zintl, R., de Vente, J. and Nighorn, A. (2001). Localization of cGMP immunoreactivity and of soluble guanylyl cyclase in antennal sensilla of the hawkmoth *Manduca sexta*. *Cell Tissue Res.* **304**, 409-421.

Stopfer, M., Bhagavan, S., Smith, B. H. and Laurent, G. (1997). Impaired odour discrimination on desynchronization of odour-encoding neural assemblies. *Nature* **390**, 70-74.

Tanoue, S., Krishnan, T., Krishnan, B., Dryer, S. E. and Hardin, P. E. (2004). Circadian clocks in antennal neurons are necessary and sufficient for olfaction rhythms in *Drosophila*. *Curr. Biol.* **14**, 638-649.

Tumlinson, J. H., Brennan, M. M., Doolittle, R. E., Mitchell, E. R., Brabham, A., Mazomemos, B. E., Baumhover, A. H. and Jackson, D. M. (1989). Identification of a pheromone blend attractive to *Manduca sexta* (L.) males in a wind tunnel. *Arch. Insect Biochem. Physiol.* **10**, 255-271.

Worster, A. S. and Seabrook, W. D. (1988). Electrophysiological investigation of diel variations in the antennal sensitivity of the male spruce budworm moth *Choristoneura fumiferana* (Lepidoptera: Tortricidae). *J. Insect Physiol.* **35**, 1-5.

Zack, C. (1979). Sensory adaptation in the sex pheromone receptor cells of saturniid moths. PhD thesis, Ludwig-Maximilians-Universität, München, Germany.

Zhou, X., Yuan, C. and Guo, A. (2005). *Drosophila* olfactory response rhythms require clock genes but not pigment dispersing factor or lateral neurons. *J. Biol. Rhythms* **20**, 237-244.

Ziegelberger, G., van den Berg, M. J., Kaissling, K.-E., Klumpp, S. and Schultz, J. E. (1990). Cyclic GMP levels and guanylate cyclase activity in pheromone-sensitive antennae of the silkmoths *Antheraea polyphemus* and *Bombyx mori*. *J. Neurosci.* **10**, 1217-1225.

Zufall, F. and Hatt, H. (1991). Dual activation of a sex pheromone-dependent ion channel from insect olfactory dendrites by protein kinase C activators and cyclic GMP. *Proc. Natl. Acad. Sci. USA* **88**, 8520-8524.

Zufall, F., Stengl, M., Franke, C., Hildebrand, J. G. and Hatt, H. (1991). Ionic currents of cultured olfactory receptor neurons from antennae of male *Manduca sexta*. *J. Neurosci.* **11**, 956-965.

Octopamine and tyramine modulate pheromone-sensitive olfactory sensilla of the hawkmoth *Manduca sexta* in a time-dependent manner

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ORIGINAL PAPER

Octopamine and tyramine modulate pheromone-sensitive olfactory sensilla of the hawkmoth *Manduca sexta* in a time-dependent manner

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Abstract In moths octopamine improved pheromone-dependent mate search time dependently. In the nocturnal hawkmoth *Manduca sexta* long-term tip recordings of trichoid sensilla were performed to investigate whether biogenic amines modulate pheromone transduction time dependently. At three Zeitgebertimes octopamine, tyramine and the octopamine antagonist epinastine were applied during non-adapting pheromone-stimulation. At ZT 8-11, during the photophase, when sensilla were adapted, octopamine and to a lesser extent tyramine increased the bombykal-dependent sensillar potential amplitude and initial action potential (AP) frequency. In addition, during the photophase, when sensilla are less able to resolve pheromone pulses, octopamine rendered pheromone responses more phasic and sensitive, and raised the spontaneous AP frequency. During the late scotophase, at ZT 22-1, when the antenna appeared maximally sensitized for pheromone pulse detection and endogenous octopamine levels are high, exogenously applied octopamine was ineffective. Epinastine blocked the pheromone-dependent AP response at ZT 8-11 and slightly affected it at ZT 22-1, while it had no effect on the sensillar potential amplitude. Epinastine decreased the spontaneous AP activity during photophase and scotophase and rendered pheromone responses more tonic in the scotophase. We hypothesize that the presence of octopamine in the antenna is obligatory for the detection of intermittent pheromone pulses at all Zeitgebertimes.

Keywords Insect olfaction · Pheromone transduction · Time-dependent differences · Octopamine · Tyramine

Abbreviations

AP	Action potential
BAL	Bombykal
EAG	Electroantennogram
ISI	Interspike interval
ISTI	Interstimulus interval
OA	Octopamine
ORN	Olfactory receptor neuron
PER	PERIOD
PSTH	Post-stimulus-time-histogram
SP	Sensillar potential
TA	Tyramine
ZT	Zeitgebertime

Introduction

Mate finding in the nocturnal hawkmoth *Manduca sexta* is mediated by species-specific, pulsatile pheromone signals emitted by female moths. Males detect the intermittent pheromone pulses with specialized trichoid sensilla located in large numbers at the leading edge of the antenna. Each sensillum is innervated by two olfactory receptor neurons (ORNs) (Sanes and Hildebrand 1976; Keil 1989; Lee and Strausfeld 1990). They are sensitive to different components of the pheromone blend, with one of them always responding to bombykal (BAL), the main sex pheromone component (Tumlinson et al. 1989; Kaissling et al. 1989).

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The calling behavior and pheromone release of female *M. sexta* moths show a diurnal distribution with the peak at the end of the scotophase (Itagaki and Conner 1988). This rhythmic pattern of calling behavior persisted under constant conditions and, thus, is controlled by a circadian pacemaker. Also, the behavior of male moths appears to be under circadian control. *M. sexta* males show highest mating activity in the scotophase (Lingren et al. 1977) and the maxima in male flight activity and in calling behavior of females are correlated in the scotophase (Sasaki and Riddiford 1984). In addition, males of other moth species, such as, *Trichoplusia ni* and *Agrotis segetum*, showed a distinct circadian rhythm in their responsiveness to pheromone with its maximum during the scotophase (Linn Jr et al. 1996; Rosén et al. 2003). Thus, the calling behavior of female moths and pheromone-dependent flight activity of male moths occur both synchronized in the scotophase (Sasaki and Riddiford 1984; Itagaki and Conner 1988; Rosén 2002; Rosén et al. 2003; Silvegren et al. 2005). The identity and location of the respective circadian pacemakers and coupling factors which modulate pheromone-dependent behavior are mostly unknown.

Octopamine (OA) an invertebrate-specific biogenic monoamine structurally related to norepinephrine acts centrally as well as peripherally as neuromodulator, neurohormone and neuromediator (Roeder 2005; Farooqui 2007). It is a good candidate for a circadian coupling factor. OA could synchronize circadian rhythms in the activity of peripheral and central neuronal circuits which control pheromone-dependent behavior. Indeed, for *M. sexta* it was shown that the concentration of OA in the hemolymph changes in a circadian rhythm, correlating with peak levels of male flight activity (Lehman 1990). Furthermore, an OA receptor was located in the antenna of *M. sexta*, but the source of OA in the antenna remained unknown, in contrast to the honeybee (Dacks et al. 2006; Schröter et al. 2007). Also, in *T. ni* the levels of OA in the hemolymph and brain change time dependently (Linn Jr et al. 1994) and are controlled by an endogenous oscillator (Linn Jr et al. 1996). In addition, injection of OA into the hemolymph of *T. ni*, *Lymantria dispar*, and *Grapholita molesta* males improved pheromone-source finding in wind tunnel experiments mostly time dependently (Linn Jr and Roelofs 1984, 1986, 1992; Linn Jr et al. 1992). This OA-dependent sensitization of male behavior might be partly due to central effects and partly due to improved peripheral pheromone detection since OA sensitized antennal sensilla in other moth species such as *Antherea polyphemus*, *Bombyx mori* and *Mamestra brassicae* (Pophof 2000, 2002; Grosmaitre et al. 2001). However, in recordings from single pheromone-sensitive sensilla it was never tested if OA influences the pheromone-transduction time dependently.

Long-term tip recordings were performed to investigate whether OA and its precursor tyramine (TA) affect pheromone detection at the periphery time dependently. OA and TA were perfused into pheromone-sensitive trichoid sensilla via the tip recording electrode at three Zeitgebertimes (ZTs: ZT 22-1, ZT 1-4 and ZT 8-11; with ZT 0 = lights on). In addition, the OA receptor antagonist epinastine (EPI) (Roeder et al. 1998) was perfused at ZT 22-1 and ZT 8-11 to block OA-mediated effects. During ZT 22-1 the transition from scoto- to photophase occurs and *M. sexta* males show high flight activity and mating behavior. Furthermore, at this time endogenous OA levels are high. At ZT 1-4, at the beginning of the photophase, moths switch from their active to their inactive phase and OA levels decline. At ZT 8-11, at the middle of the photophase, most moths are resting and endogenous OA levels are low. Pheromone responses to the main pheromone component BAL and spontaneous action potentials (APs) recorded between stimulations were evaluated.

Materials and methods

For the recordings male *M. sexta* moths (Johannson) (Lepidoptera: Sphingidae) were used which were reared from eggs in our laboratory at the University of Marburg. Larvae were fed on an artificial diet (modified after Bell and Joachim 1976) and held under long-day photoperiod conditions (L:D 17:7 h) at 24–27°C and 40–60% relative humidity. The preparation, recording conditions and digitization of the data were described before (Flecke et al. 2006). To be able to record during the scotophase red light emitting LEDs with a narrow frequency spectrum were used for the illumination of the preparation and the setup (Kingbright LKR 530100, Kingbright Electronics, Issum, Germany). It was shown that monochromatic light with a wavelength above 600 nm does not function as a Zeitgeber and failed to synchronize circadian rhythms of moths (Pittendrigh et al. 1970). All light sources emitting light in a frequency spectrum which might affect circadian rhythms of *M. sexta* were eliminated. After taking the moths out of the rearing facility at ZT 21 they were always kept under red light. Thus, the length of the scotophase was not affected. All experiments in the photophase were performed with room lights switched on. The experiments started either at ZT 22, the end of the scotophase, at ZT 1 which is the beginning of the photophase 1 h after lights are switched on, or at ZT 8 which is at the middle of the photophase. In experiments starting at ZT 22 the transition from scoto- to photophase was simulated by switching the room lights on at ZT 0 (Fig. 1a).

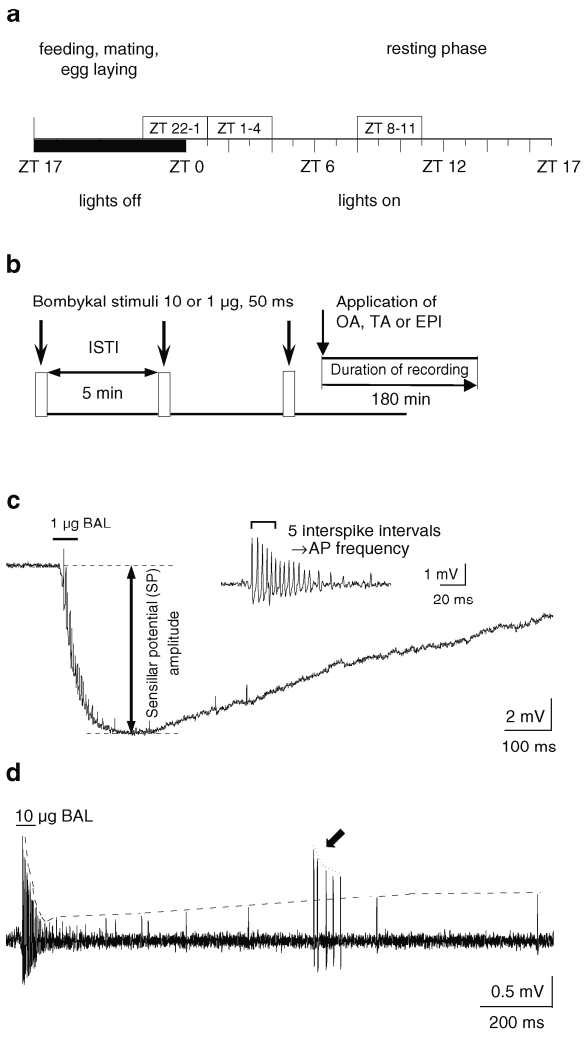


Fig. 1 **a** The recordings were performed during three ZTs, *ZT 0* = lights on. **b** Non-adapting stimulus-protocol: 50 ms long stimuli of 10 or 1 µg BAL per filter paper were applied with an interstimulus-interval (*ISTI*) of 5 min over a period of 180 min. OA or TA at a concentration of 1 mmol l^{-1}, or EPI with concentrations of 1 or 0.1 mmol l^{-1} were applied via the recording electrode. **c** The pheromone response is characterized by two main parameters: A non-filtered response to a 50-ms stimulus of 1 µg BAL. APs are superimposed on the negative deflection of the transepithelial potential, the SP response. The maximal SP amplitude is measured between the baseline before the response and the negative peak of the SP. For the analysis of all parameters describing the SP, the responses were lowpass filtered at 50 Hz. For the analysis of APs, the lowpass-filtered response is subtracted from the original trace (Pseudo-highpass-filtering), yielding a *straight baseline*. The initial AP frequency is computed over the first five ISIs. **d** Pseudo-highpass-filtered response to a 50-ms stimulus of 10 µg BAL. The amplitudes of the large APs are reduced after strong BAL stimuli and regain their original amplitude in the course of several seconds (*dashed envelope*). A burst of spontaneous APs of the non-BAL cell occurred (*filled arrow*) after the response. APs of the non-BAL cell can be separated from the BAL-APs by their lower amplitude during spontaneous activity or different amplitude during the BAL-response (modified after Dolzer et al. 2003; Flecke et al. 2006)

Application of OA, TA and EPI

Drug application to single trichoid sensilla of intact male *M. sexta* by inclusion of water soluble agents in the recording electrode were performed as described before (Flecke et al. 2006). The included agents such as OA diffused slowly into the sensillum lymph. In 3-h long control recordings no decline due to damage or adaptation was observed when sensilla were stimulated with strong but short stimuli in intervals of 5 min. Note that in controls at ZT 8-11 and to a much lesser degree at ZT 1-4 (Flecke et al. 2006) the number of APs during the first 100 ms of the response decreased; however the evaluated parameters SP amplitude and initial AP frequency, which describe the strength of responses, remained stable over the recording duration. It was shown before that this change in the response kinetics occurs time dependently and is not an artifact due to damage of the sensillum (Flecke et al. 2006). Also, long-term recordings of unstimulated sensilla in situ revealed no damage of the sensilla when continued for up to several days (Dolzer et al. 2001). The sensillum lymph ringer of the recording electrode passively perfused the sensillum lymph cavity. Therefore, altered sensillum lymph ringer solutions with a concentration of 1 mmol l^{-1} OA or TA were employed. EPI was used in the same way in a concentration of 1 or 0.1 mmol l^{-1}. These concentrations were selected to obtain significant perfusion during the first hour of the recording and are in the range of hemolymph concentrations employed in previous studies (Grosmaitre et al. 2001; Dolzer et al. 2001; Pophof 2000, 2002). All ringer solutions were prepared with *N*-(2-hydroxyethyl)-piperazine-*N*′-(2-ethane sulfonic acid) (HEPES; all chemicals from Sigma, Deisenhofen, Germany). The pH of the sensillum lymph and hemolymph ringer was adjusted to 6.5. Osmolality was adjusted with mannitol to 475 mosmol l^{-1} for the sensillum lymph ringer and to 450 mosmol l^{-1} for the hemolymph ringer.

Pheromone stimulation

The pheromone stimulations were performed as described before (Flecke et al. 2006). Synthetic bombykal (BAL, (*E*,*Z*)-10,12-hexadecadienal) generously provided by T. Christensen (Tucson, AZ, USA) and J. Krieger (Stuttgart, Germany) was applied in a non-adapting stimulation protocol (10 or 1 µg BAL, stimulus duration 50 ms) every 5 min for 180 min of the recording duration (Fig. 1b).

Acquisition protocols

Each sensillum trichodeum in *M. sexta* antennae houses two ORNs. Both ORNs are sensitive to different components of the pheromone blend, only one of the ORNs

responds to stimulation with the main component BAL. The APs of both ORNs can be distinguished by their amplitude (Dolzer et al. 2001). The ORNs generate spontaneous APs with a mean frequency of 0.53 ± 0.13 Hz for small and 0.39 ± 0.06 Hz for large APs, whereas 45.7 ± 2.1 and $47.4 \pm 2.7\%$, respectively of the APs occur in bursts (data from 66 recordings, 438 segments of 10 min duration) (Dolzer et al. 2001). The spontaneous activity between stimulations was recorded in intervals of approximately 5 min with a sampling frequency of 19.6 kHz (Clampex 8, fixed-length events). Each AP triggered a sweep of 12.75 ms duration, and the highpass-filtered signal served only as a trigger channel. All the analyses were performed using the direct-current-coupled signal. The pheromone responses were recorded in sweeps of approximately 5 s duration at a continuous sampling rate of 20 kHz (Clampex 8, Episodic Stimulation Mode).

Data analysis

Both the recordings of the pheromone responses and of the spontaneous activity were evaluated using the Microsoft Excel Add-in XtraCell (Dolzer 2002) and Clampfit 8. For the analysis of the SP, the responses were lowpass filtered at a cutoff frequency of 50 Hz (Clampfit, Gaussian filter). Subsequently, different parameters of the SP response were evaluated as described before (Flecke et al. 2006). This includes the maximal SP amplitude (Fig. 1c). For the analysis of the APs (Fig. 1c insert), the lowpass-filtered trace was pseudo-highpass filtered as described before (Flecke et al. 2006). The time and positive amplitude of every AP occurring during the 5-s long sweep recorded in response to the stimulation were counted. When APs of both amplitude classes were observed in the response, they were analyzed separately. The AP response was evaluated as described before (Flecke et al. 2006), whereas the main parameter was the AP frequency calculated over the first five interspike intervals (ISIs) according to $f = n \times 1{,}000/\Sigma\text{ISI}$ (n = number of ISI). The evaluated response parameters showed a high variability between the recordings, thus each time course was normalized to the first value of the recording. Afterward the data for each parameter of all associated recordings was binned to 5 min intervals. To compare the normalized and binned time courses of OA, TA, EPI and associated control recordings of each time slot statistically the Mann–Whitney test or the Student's t test were used, depending on the distribution of the data. For the statistical analysis of changes over the recording duration of a parameter its time course was divided into three or four intervals and then analyzed with a One-way ANOVA followed by the Tukey HSD post hoc test or with the Mann–Whitney test. In addition the absolute values of the AP frequency in responses to pheromone stimulation were analyzed. Therefore, the mean was calculated over the 5-ISI AP frequencies in all responses for each ZT. Afterward, the complete data set for each ZT was compared to other ZTs using the Mann–Whitney test. To analyze the AP amplitude reduction APs of three consecutive responses in an interval of 1 min before to 2 min after the stimulus were counted and binned to 10 ms intervals. Changes in the amplitude reduction were analyzed by the calculation of the ratio of the maximal and minimal AP amplitude. To evaluate changes in the distribution of APs in responses to stimulation with BAL the APs of all responses during three intervals (0–20, 80–100 and 160–180 min of the recording) were added up, binned to 10 ms intervals and plotted as post-stimulus time histograms (PSTHs), with $t = 0$ being the start of the DC response. PSTHs of different ZTs were compared using the Mann–Whitney test. In addition the APs occurring during the first 100 ms of the responses were evaluated and the mean of this parameter was binned to 5 min intervals. Changes in the time course of this parameter were compared by dividing it into three portions followed by a One-way ANOVA with the Tukey HSD post hoc test or with the Mann–Whitney test, depending on the distribution of the data.

Analysis of spontaneous APs and bursts

For the evaluation of the spontaneous APs, the two spike classes had to be sorted. Spike sorting of spontaneous APs and calculation of bursts were performed as described before (Dolzer et al. 2001). APs separated by an ISI of not more than 50 ms were defined as part of a burst. The AP amplitude reduction within bursts did not allow a threshold-based AP sorting. Since the peak-to-peak amplitude of APs in bursts continuously decrease, the following AP was only considered as part of the burst when the peak-to-peak amplitude was smaller than the amplitude of the previous AP. After separation of APs, the ISIs for each 5 min interval were determined. Then, the percentage of APs in bursts, the mean of APs per burst and the coefficient of variation (CV) of the ISIs (CV = SD/mean) were calculated. The CV was used as a burst criterion (Rospars et al. 1994; Dolzer et al. 2001). In a sequence of events that occur independently of preceding events, i.e., in a Poisson process, the CV of the intervals between every two successive events is equal to 1. Thus, a CV significantly unequal to 1 indicates that the APs are not randomly distributed. The time courses of the percentage of APs in bursts and the mean of APs per burst were normalized to the first value and then binned to 5 min intervals. The data of the OA, TA and EPI recordings were compared with the Mann–Whitney test or the Student's t test. Changes in each time course were analyzed with a One-way ANOVA followed by the Tukey HSD post hoc test. All statistical calculations were done with SPSS (version 11) (SPSS Inc., Chicago, IL, USA).

Results

In extracellular tip recordings of single trichoid sensilla of the hawkmoth *M. sexta* time-dependent effects of OA, TA and EPI on BAL-stimulated ORNs were investigated. It was tested whether the application of biogenic amines at different ZTs and, thus, during different endogenous concentrations of OA in the hemolymph affect moths ORNs time dependently. In addition the OA-receptor antagonist EPI was used to block OA-receptors at ZT 22-1 and ZT 8-11. Ringer solutions with a concentration of 1 mmol l^{-1} OA or TA and 1 mmol l^{-1} or 0.1 mmol l^{-1} EPI were applied via perfusion with the recording electrode. The 3-h long recordings were performed from ZT 22-1, ZT 1-4 and ZT 8-11 (Fig. 1a). The olfactory sensilla were stimulated every 5 min with 10 or 1 µg of BAL by utilizing a non-adapting stimulation protocol (stimulus duration 50 ms; Fig. 1b). To obtain information about the targets of the amines in the signal transduction cascade, different parameters of the SP and AP response of the recordings were evaluated (Fig. 1c, d). In addition, the effects of OA, TA and EPI on spontaneous APs recorded between the stimulations were investigated.

When comparing a response from the beginning and the end of a single recording at ZT 8-11, an OA-dependent increase in the initial AP frequency and SP amplitude were observed (Fig. 2a, b). Furthermore, the AP response from the end of the recording was more phasic (Fig. 2b). In control recordings at ZT 22-1, ZT 1-4 and ZT 8-11, the normalized mean of none of the parameters describing the SP or AP response including the normalized SP amplitude and AP frequency showed significant changes throughout the 3–h long recordings (Fig. 3). Note that in the controls at ZT 8-11 (Fig. 5) and to a much lesser degree at ZT 1-4 (Flecke et al. 2006), but not at ZT 22-1 (Fig. 9) the number of APs during the first 100 ms of the response decreased over the recording duration. However, the evaluated parameters SP amplitude and initial AP frequency, that describe the strength of the initial response, remained stable over the recording duration in all three analyzed time slots. It was shown previously that this change in the response kinetics of the controls occurs in a time-dependent manner and is not an artifact due to damage of the sensillum (Flecke et al. 2006).

The normalized and averaged SP amplitude and AP frequency were analyzed for each time slot and different time-dependent effects were observed. When comparing the time course of the SP amplitude of OA recordings with the associated controls (Mann–Whitney test, $P < 0.001$ and Student's t test $P < 0.001$) it was observed that the application of 1 mmol l^{-1} OA increased the SP amplitude significantly at ZT 1-4 and ZT 8-11 (Fig. 3a). Also, when comparing the intervals in each affected time course for ZT 1-4 and ZT 8-11 (ANOVA and Tukey HSD post hoc test; $\alpha = 0.01$, $P < 0.01$ or Mann–Whitney test, $P < 0.001$) a significant rise in the SP amplitude was measured over the recording duration (Fig. 3a). The increase at ZT 1-4 was 9.74× weaker than in recordings at ZT 8-11. At ZT 22-1 no OA-dependent effects on the SP amplitude were found (Fig. 3a). In addition OA significantly increased the initial AP frequency at ZT 8-11 (Fig. 3b). Significant differences in the time course of the AP frequency were found both in the comparison between OA and control recordings (Mann–Whitney test, $P < 0.001$) and between the intervals of the affected time course (Mann–Whitney test, $P < 0.01$). In contrast to the effects on the SP amplitude the AP frequency increased only over the first 30 min of the time course and, then, reached a plateau. At ZT 22-1 and ZT 1-4

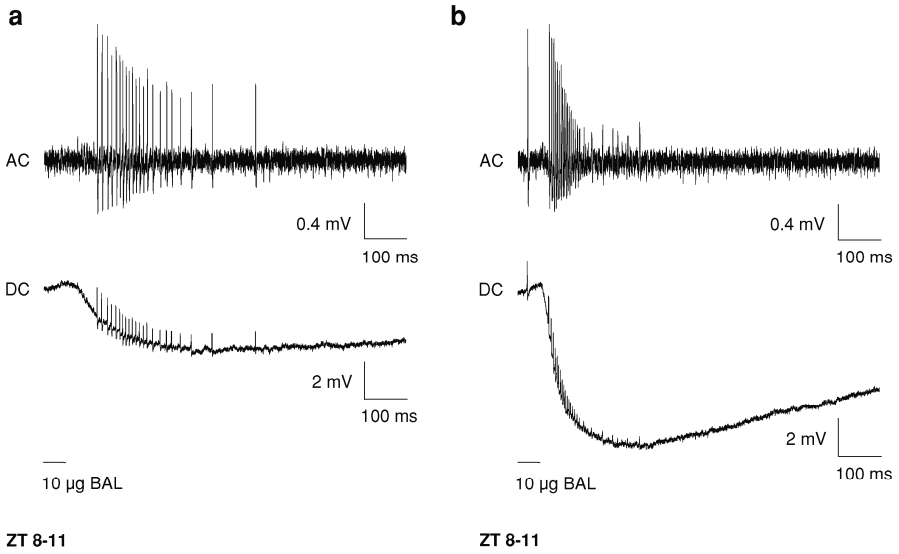
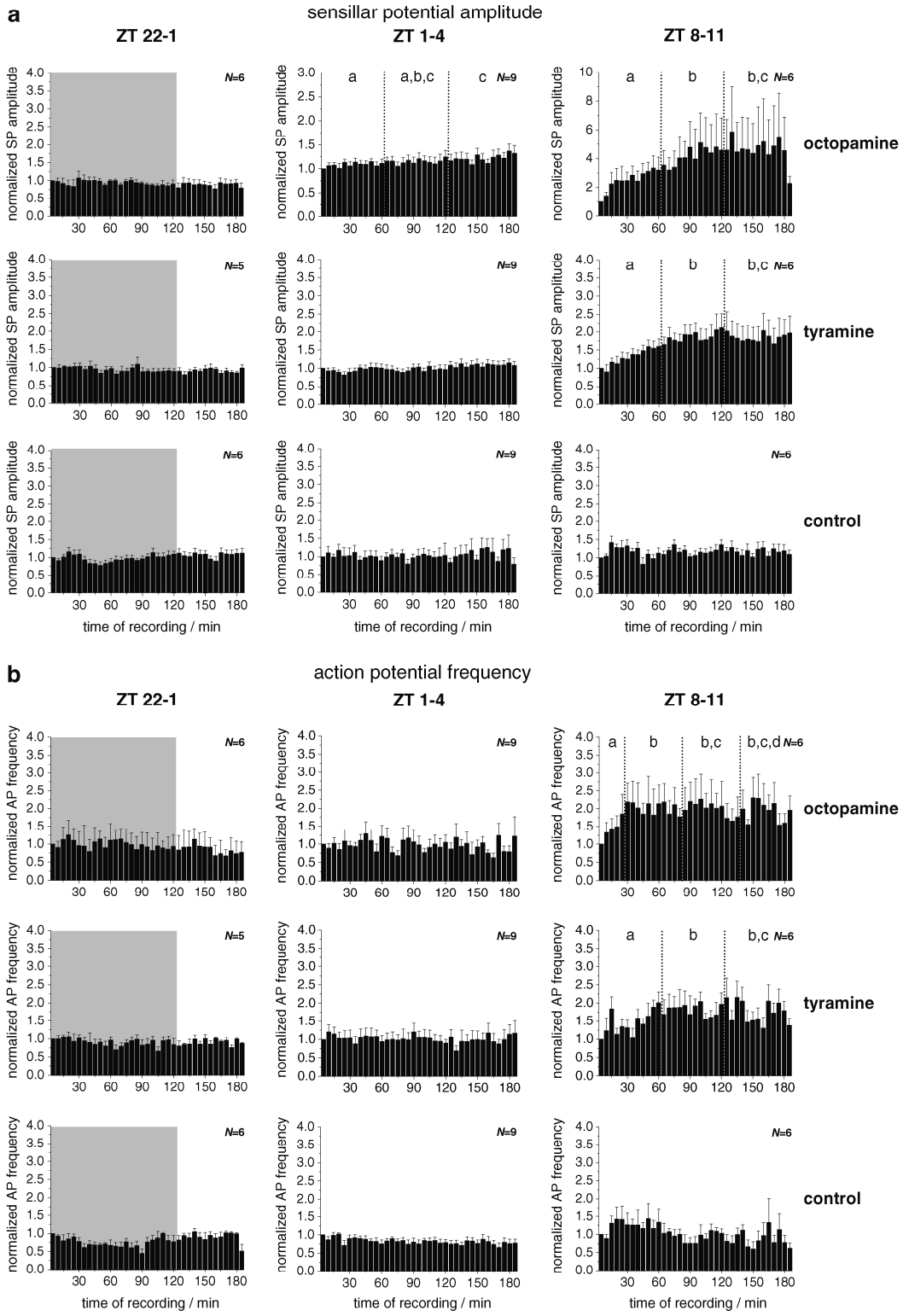


Fig. 2 Comparison of single responses to stimulation with 10 µg BAL from the beginning (**a**) and the end of a 3-h recording (**b**) at ZT 8-11. Sensillum lymph perfusion with 1 mmol l^{-1} OA increased both the SP amplitude (3.08–8.41 mV) and the initial AP frequency (95–213 Hz), and rendered the AP response more phasic. Also, the latency to the occurrence of the first AP was reduced. *AC* pseudo-high-pass filtered signal, *DC* non-filtered signal


a
sensillar potential amplitude
ZT 22-1
ZT 1-4
ZT 8-11
normalized SP amplitude
N=6
N=5
N=9
a
a,b,c
c
b
b,c
octopamine
tyramine
control
time of recording / min
b
action potential frequency
normalized AP frequency
b,c,d

◀ **Fig. 3** The normalized and binned parameters SP amplitude (**a**) and AP frequency (**b**) over 185 min for recordings with OA and TA application at ZT 22-1, ZT 1-4 and ZT 8-11. Values are mean + SEM. OA significantly increased the SP amplitude at ZT 1-4 and ZT 8-11. In addition, TA increased the SP amplitude but only at ZT 8-11. The increases were significant in comparison to the respective controls (Mann–Whitney test, $P < 0.001$ or Student's t-test $P < 0.01$) and when comparing the three intervals within each time course (separated by *dotted lines*). *Different lower case letters* denote significant differences between tested groups of means (Mann–Whitney test, $P < 0.001$ or ANOVA and Tukey HSD post hoc test; $\alpha = 0.01$, $P < 0.01$). The OA-dependent increase of the SP amplitude at ZT 8-11 was 9.74× stronger than at ZT 1-4. The TA-dependent increase was 2.5× weaker than the OA-dependent increase at ZT 8-11. The AP frequency was significantly increased by OA and TA at ZT 8-11 but not at ZT 1-4 (ANOVA and Tukey HSD post hoc test; $\alpha = 0.001$, $P < 0.001$ or Mann–Whitney test, $P < 0.001$), whereas the AP frequency increased only over the first 30 respectively 60 min and then, remained at a plateau. At ZT 22-1 no OA- or TA-dependent effects were found (*gray area* = scotophase)

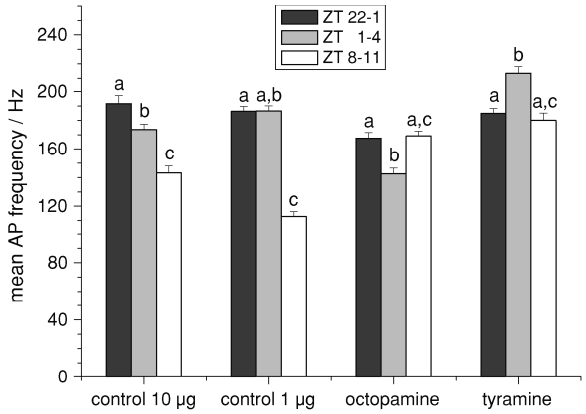


Fig. 4 The mean AP frequency (+SEM) calculated over all responses recorded during each of the three ZTs. In both control recordings with 10 and 1 µg BAL stimulation the mean AP frequency was decreased significantly at ZT 8-11 compared to ZT 22-1 (Mann–Whitney test, $P < 0.001$). In contrast OA and TA antagonized this decrease in the mean AP frequency, since no significant differences were found between ZT 22-1 and ZT 1-4. *Different lower case letters* denote significant differences between tested groups of mean values

no OA-dependent effects on the AP frequency were found (Fig. 3b).

The application of TA significantly increased the SP amplitude at ZT 8-11 only (Fig. 3a). The significance of the increase was confirmed in the comparison of the TA and the associated control recordings (Mann–Whitney test, $P < 0.001$) and when comparing the three intervals of the subdivided time course of TA recordings (Mann–Whitney test, $P < 0.001$). As in OA recordings the SP amplitude increased gradually during the first 2 h of the recordings and then reached a plateau. When comparing OA- and TA-dependent effects on the SP amplitude at ZT 8-11 the OA-dependent increase was 2.5× stronger than the TA-dependent rise.

In addition, TA also significantly increased the AP frequency at ZT 8-11 only (Fig. 3b) in both the comparison of TA and control recordings (Student's t test, $P < 0.01$), and also within the time course of the TA-dependent changes (ANOVA and Tukey HSD post hoc test; $\alpha = 0.01$, $P < 0.01$). When comparing the OA and TA recordings at ZT 8-11 it was observed that the increase in the AP frequency of TA-perfusions was more unstable but similar in amplitude (Fig. 3b). No TA-dependent effects were found for recordings at ZT 22-1 and ZT 1-4 (Fig. 3a, b).

Furthermore, OA and TA affected the mean AP frequency (Fig. 4; Table 1) calculated over all pheromone responses recorded during the same ZT. The mean AP frequency represents the mean of the absolute values and, thus, is directly proportional to the pheromone sensitivity. The mean AP frequency at ZT 22-1 was in the same range for all recordings, confirming similar recording conditions (Table 1). In control recordings employing 10 µg BAL the mean AP frequency was significantly decreased at ZT 1-4 and ZT 8-11 when compared to ZT 22-1 (Mann–Whitney test, $P < 0.001$). In accordance, also in controls with 1 µg BAL stimulation a significant decrease was found at ZT 8-11. In contrast, the application of 10 mmol l^{-1} OA or TA antagonized this decrease, since no significant differences were found in the comparison of the mean AP frequency between ZT 22-1 and ZT 8-11. In addition, at ZT 1-4 a significant decrease was found in OA recordings and a significant increase was measured in TA recordings (Mann–Whitney test, $P < 0.001$). The mean SP amplitude showed no correlation to time-dependent changes in the mean AP frequency (data not shown).

Effects on the kinetics of the AP response

Time-dependent differences in the distribution of APs in response to stimulation with BAL in recordings with OA, TA and in control recordings were discovered. To analyze

Table 1 Mean AP frequency (±SEM) in responses to stimulation with BAL for three ZTs

	Control 10 µg BAL	Control 1 µg BAL	Octopamine	Tyramine
ZT 22-1	191.65 ± 5.54 (n = 188)	186.38 ± 3.45 (n = 255)	167.51 ± 4.39 (n = 217)	184.77 ± 3.58 (n = 179)
ZT 1-4	173.57 ± 3.87 (n = 283)	186.61 ± 3.51 (n = 216)	142.5 ± 4.19 (n = 256)	213.51 ± 4.44 (n = 273)
ZT 8-11	143.37 ± 4.68 (n = 191)	112.87 ± 3.29 (n = 251)	169.19 ± 3.47 (n = 211)	180.01 ± 5.14 (n = 218)

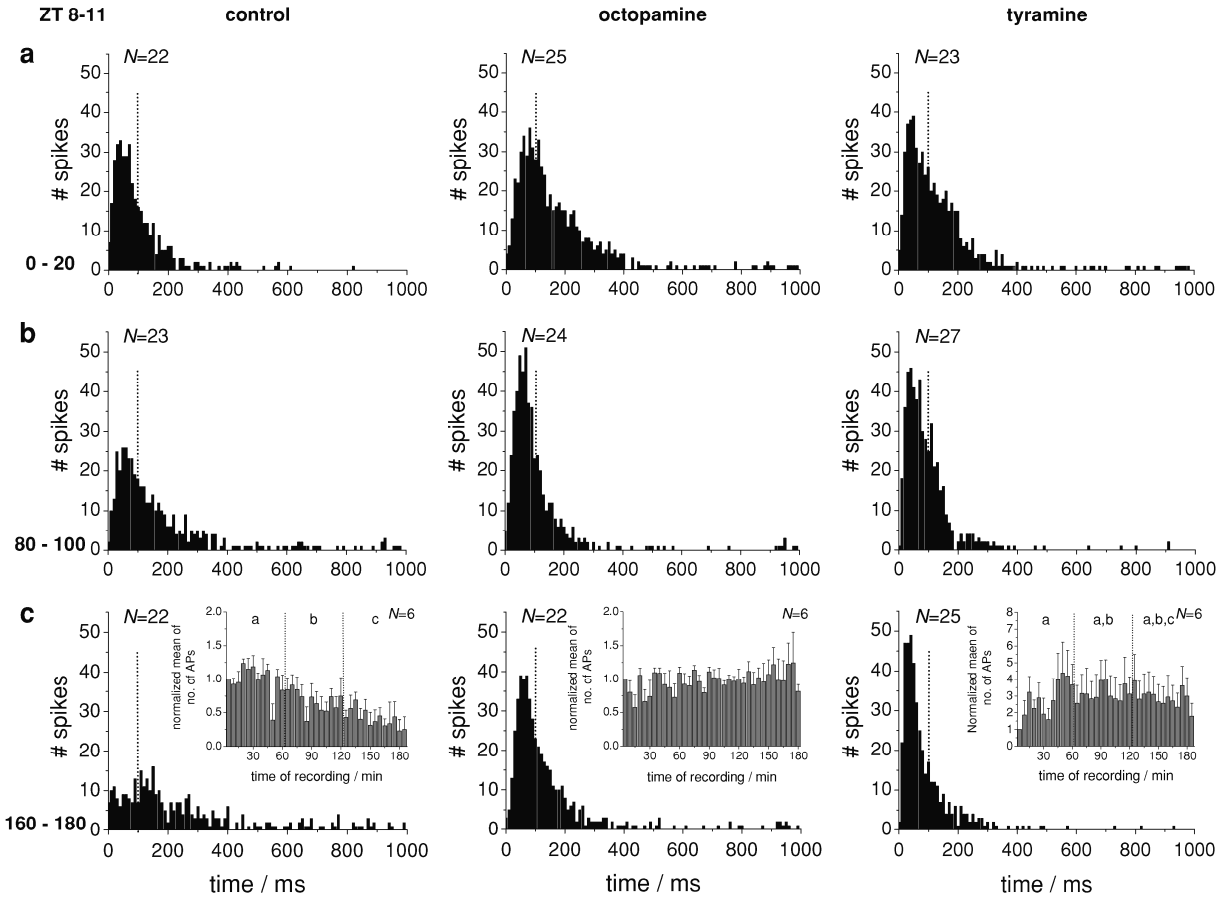


Fig. 5 The distribution of APs in responses to stimulation with 10 µg BAL for recordings at ZT 8-11. PSTHs (binwidth = 10 ms) for the beginning (**a**; 0–20 min), middle (**b**; 80–100 min) and end of the recordings (**c**; 160–180 min). In the controls the first phasic part of the response became more tonic over the course of the recordings. Also, the numbers of APs over the first 100 ms (insert in **c**) decreased significantly (Flecke et al. 2006). *Different lower case letters* denote significant differences between tested groups of means which are separated by *dashed lines* in the insert (ANOVA and Tukey HSD post hoc test; $\alpha = 0.01$, $P < 0.01$). OA counteracted the shift from a phasic to a tonic response pattern. Also, the number of APs over the first 100 ms of the response did not decline. For TA application a shift to more phasic responses with recording duration was observed. The apparent increase in the APs over the first 100 ms, however, was not significant (Mann–Whitney test, $P > 0.01$)

the distribution of APs occurring during the first 1,000 ms of the response, the APs were binned to 10 ms intervals and plotted as PSTHs (Fig. 5) for the beginning (0–20 min), middle (80–100 min) and end (160–180 min) of the recordings. As shown before, control recordings at ZT 8-11 expressed time-dependent effects in the distribution of APs (Flecke et al. 2006). The responses changed from a phasic to a more tonic pattern over the recording duration at ZT 8-11, but not at ZT 22-1 (Fig. 5). Also, the number of APs occurring during the first 100 ms of the BAL responses decreased significantly over the course of the recordings (Fig. 5, insert in c) (ANOVA and Tukey HSD post hoc test; $\alpha = 0.01$, $P < 0.01$). The application of 1 mmol l^{-1} OA or TA antagonized this shift to more tonic responses (Mann–Whitney test, $P < 0.01$). When comparing the PSTHs of OA and TA recordings with the control it was found that the responses at the middle and end of the recordings were more phasic under the influence of OA and TA (Fig. 5). In addition the numbers of APs in the first 100 ms did not decrease (Fig. 5, inserts in c) in OA recordings. The TA-dependent rise in the number of APs in the first 100 ms was not significant due to high variability (Fig. 5, insert in c) (Mann–Whitney test, $P > 0.01$). In recordings at ZT 22-1 and ZT 1-4 no OA- or TA-effects on the AP distribution were found (data not shown).

Effects on the amplitude reduction

Typically the peak-to-peak AP amplitude in responses to BAL stimuli of a higher dosage is reduced during the phasic portion of the response (Fig. 1c, d). The time courses of the positive AP amplitude at the beginning, middle and end

of a single recording with 1 mmol l^{-1} OA application were compared (Fig. 6a). It was apparent that under the influence of OA the amplitude reduction increased over the course of the recordings. In contrast, there were no strong changes in the associated control recordings. The ratio of the maximal and minimal positive AP amplitude in responses of all recordings at ZT 8-11 was increased by OA (Fig. 6b). This rise in the ratio reflects an increase in the AP amplitude reduction. This OA-dependent increase was significant in comparison to the controls (Mann–Whitney test, $P < 0.001$). The application of TA was followed by an increase in the ratio in two recordings. However, in the rest of the recordings the TA application caused a decrease, leading to two different populations. No significant difference was found in comparison to the controls (Mann–Whitney test, $P < 0.01$). At ZT 22-1 and ZT 1-4 OA and TA had no effect on the AP amplitude reduction (data not shown).

Effects on spontaneous APs

The application of OA also affected spontaneous APs recorded in the 5-min intervals between the responses. In recordings at ZT 8-11 the perfusion of the sensillar lymph with 1 mmol l^{-1} OA significantly increased the mean spontaneous AP frequency, the percentage of APs occurring in bursts and the number of bursts (Fig. 7). In addition the average spikes per burst were slightly (2.35–2.65 APs per burst) but significantly increased by application of OA (data not shown). The OA-dependent time courses were compared to the respective controls (Mann–Whitney test, $P < 0.001$) and within the intervals of the time courses (ANOVA and Tukey HSD post hoc test; $\alpha = 0.01$, $P < 0.01$ or Mann–Whitney test, $P < 0.001$). At ZT 22-1 a weak but significant increase in the number of bursts was found, but no effect on the mean spontaneous AP frequency was detected (data not shown). Under the influence of 1 mmol l^{-1} TA the evaluated parameters remained stable over the recoding duration (Fig. 7); however, the time courses of the TA recordings were not significantly different from the control recordings. In controls at ZT 22-1 and ZT 8-11 both the spontaneous AP frequency and the number of bursts were significantly decreased (Mann–Whitney test, $P < 0.001$). At ZT 1-4 the application of OA and TA did not affect the generation of spontaneous APs.

Effects of epinastine

The application of the OA receptor antagonist EPI showed several time-dependent effects. At ZT 8-11 (Fig. 8a) the application of 0.1 mmol l^{-1} EPI decreased the initial AP frequency in responses to stimulation with BAL within 65 min to 0 (Mann–Whitney test, $P < 0.01$). The EPI-dependent decrease of the SP amplitude was not significant (Fig. 8a). Although at ZT 22-1 a higher EPI concentration of 1 mmol l^{-1} was used EPI had no effect on the SP amplitude but reduced the AP frequency significantly (Fig. 8a; Mann–Whitney test, $P < 0.01$). In addition EPI significantly decreased the mean spontaneous AP frequency at ZT 8-11 (0.1 mmol l^{-1}) and also at ZT 22-1 (1 mmol l^{-1}) (Fig. 8b; Mann–Whitney test, $P < 0.001$). In the controls at ZT 22-1 the mean AP frequency was lowered abruptly and significantly after 25 min (Mann–Whitney test, $P < 0.001$). In addition, in the controls at ZT 8-11 a significant decrease of the spontaneous activity (ANOVA and Tukey HSD post hoc test; $\alpha = 0.001$, $P < 0.001$) was observed which was 2.96× weaker than the EPI effect at ZT 8-11. The EPI-dependent decreases at ZT 22-1 and ZT 8-11 were significant when compared to the respective controls (Mann–Whitney test, $P < 0.01$). EPI also affected the AP distribution in responses to stimulation with BAL. At ZT 22-1 (Fig. 9) the application of 1 mmol l^{-1} EPI shifted the response characteristics from phasic to tonic responses and decreased the number of APs in the first 100 ms significantly (ANOVA and Tukey HSD post hoc test; $\alpha = 0.01$, $P < 0.01$). Also at ZT 8-11 the application of 0.1 mmol l^{-1} EPI significantly decreased the APs during the first 100 ms of the response (Fig. 10) when compared to the respective control (Mann–Whitney test, $P < 0.01$). The decrease was much stronger and faster compared to the EPI effect at ZT 22-1.

Discussion

To search for time-dependent differences in the effects of biogenic amines on the pheromone transduction cascade OA and its precursor TA were perfused at different ZTs during long-term tip recordings of single trichoid sensilla of male *M. sexta*. Both OA and TA time dependently affected responses to stimulation with the main pheromone-component BAL which was applied in a non-adapting stimulation protocol. During the photophase, at ZT 8-11, OA and to a lesser extent also TA increased the pheromone-dependent SP amplitude, increased the initial AP frequency and rendered the pheromone responses more phasic in comparison to the control. OA also increased the SP amplitude at ZT 1-4, but to a lesser extent as compared to ZT 8-11. Furthermore, an endogenous shift to a lower mean AP frequency in responses to BAL stimulation was found in control recordings at ZT 8-11. The application of OA and TA antagonized this decrease in pheromone sensitivity during the photophase. In addition, at ZT 8-11 OA, but not TA, increased the AP amplitude reduction, the spontaneous AP frequency and the number of spontaneous bursts. During the scotophase, at ZT 22-1, exogenously applied OA also

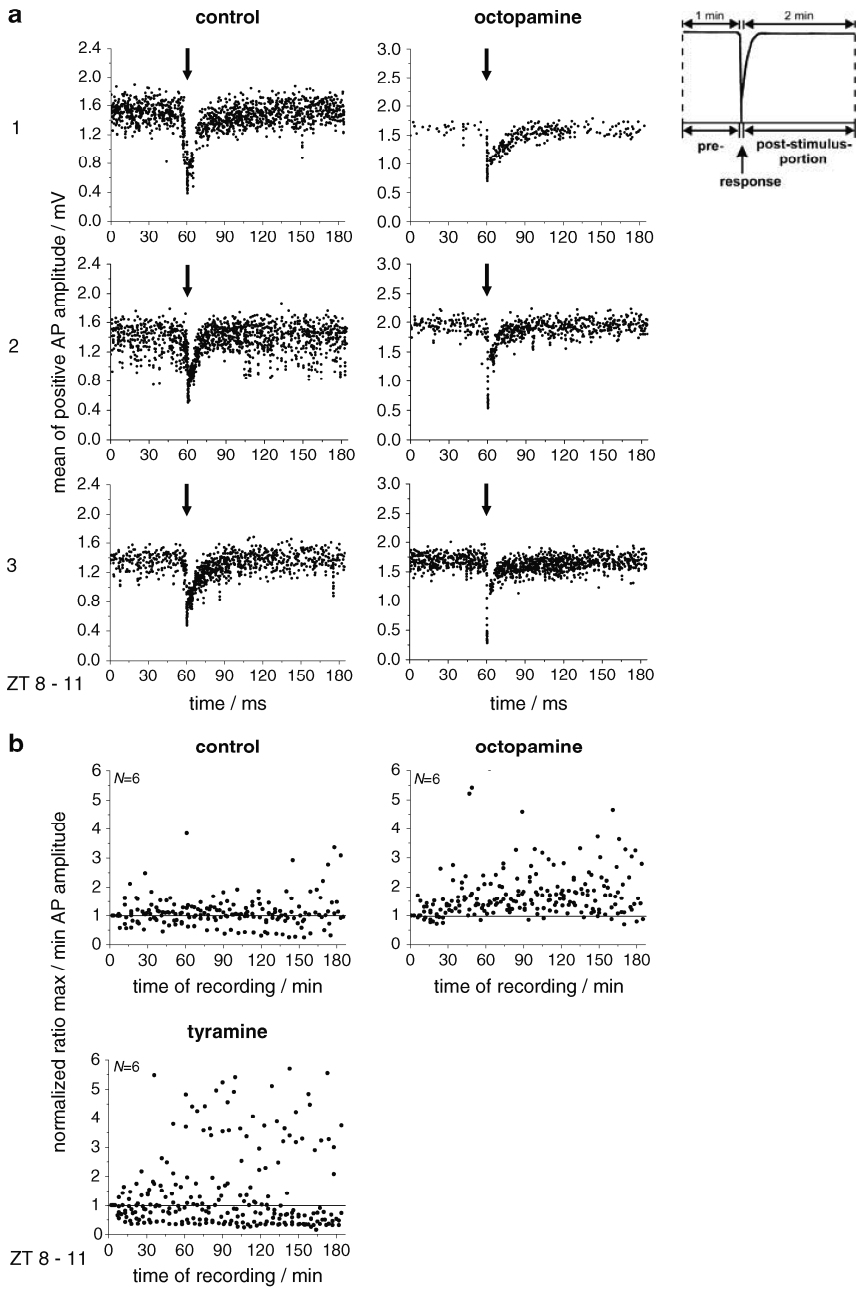


Fig. 6 Amplitude reduction of APs is enhanced with OA. **a** The AP amplitude reduction for the beginning (1), the middle (2) and the end (3) of one control and one recording with OA perfusion at ZT 8-11. Plots show the mean of the positive amplitude of APs that occurred during an interval of 1 min before to 2 min after the stimulation. *Arrows* indicate the time of stimulation. Each plot consists of the binned (binwidth = 10 ms) and averaged AP amplitudes of three consecutive responses to stimuli with 10 µg BAL. In the control recordings no strong changes in the amplitude reduction occurred, while OA enhanced the amplitude reduction. Also the number of APs in this recording increased with the recording duration. Due to the non-linear change of the BAL-AP amplitude in the post-stimulus portion BAL- and non-BAL APs could not be distinguished. **b** Normalized ratio between the maximal and minimal positive AP amplitude of a response as a mean of the strength of the amplitude reduction. Each recording was normalized to the first value. Values >1 represent an increase in the reduction of the AP amplitude. In control recordings from ZT 8-11 no clear change was observed, while OA enhanced the amplitude reduction in all recordings. The OA-dependent increase was significant in comparison to the controls (Mann–Whitney test, $P < 0.001$). The application of TA did not have a clear effect. In two recordings the amplitude reduction increased, however, in the rest of the recordings the amplitude reduction was decreased leading to two populations. The TA effect was not significant (Mann–Whitney test, $P > 0.01$)

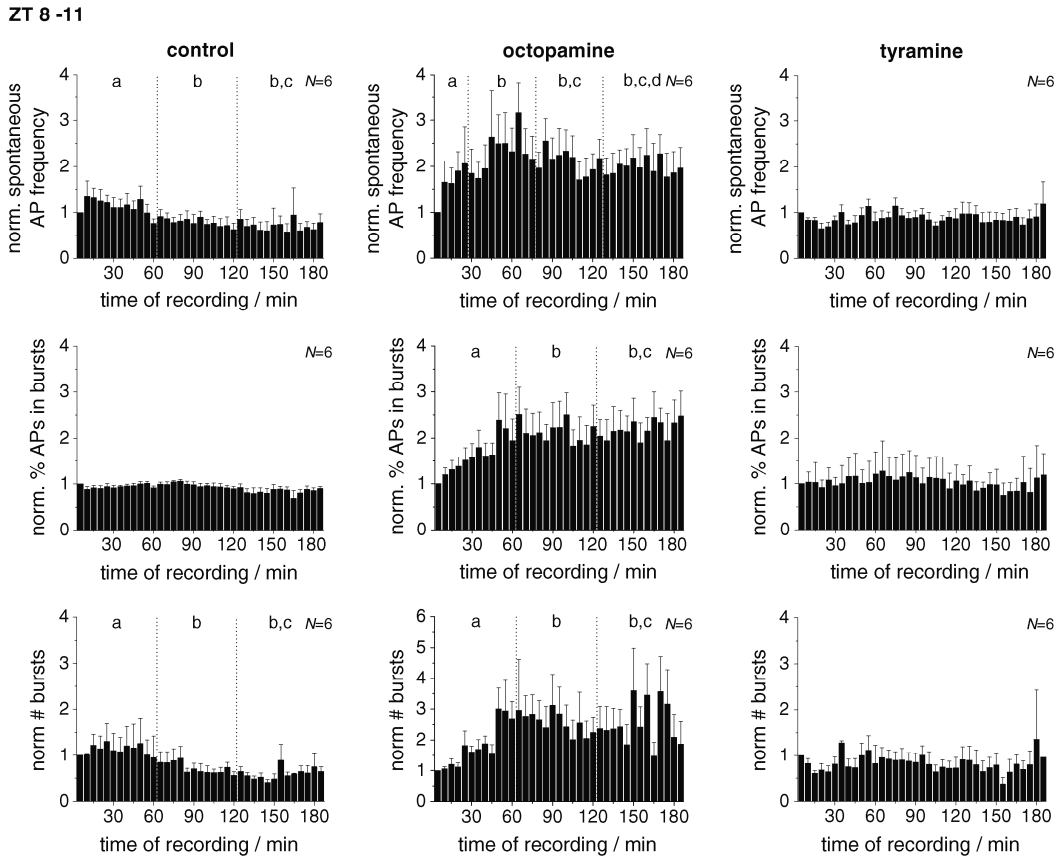


Fig. 7 Octopamine also affected the generation of spontaneous APs. The application of 1 mmol l^{-1} OA at ZT 8-11 increased the mean AP frequency, the percentage of APs occurring in bursts and the number of bursts. The increases were significant with respect to the controls (Mann–Whitney test, $P < 0.001$) and with respect to the different intervals of the time course (ANOVA and Tukey HSD post hoc test; $\alpha = 0.01$, $P < 0.01$ or Mann–Whitney test, $P < 0.001$). *Different lower case letters* denote significant differences between tested groups of mean values which are separated by *dashed lines*. In addition in the controls the mean AP frequency and the number of bursts were significantly decreased (Mann–Whitney test, $P < 0.001$). In recordings with TA application the evaluated parameters remained stable over the recording duration, however no significant differences were found in comparison to the control recordings

increased the number of spontaneous bursts but had no significant effect on the mean spontaneous AP frequency. The OA antagonist EPI almost completely abolished the pheromone-dependent AP frequency at ZT 8-11, decreased it to a lower extent at ZT 22-1, and diminished the spontaneous AP frequency at both times. In addition, EPI shifted the responses from a phasic to a tonic response pattern and decreased the APs during the first 100 ms of the pheromone response both at ZT 22-1 and ZT 8-11. However, it never affected pheromone-dependent SP amplitudes.

OA and TA disadapt peripheral ORNs during the photophase

Our data indicated for the first time that OA might be obligatory for pheromone responses during the photophase, while in the scotophase an additional mechanism guaranteed high sensitivity of pheromone responses. In accordance with hemolymph injection of OA in *B. mori* (Pophof 2002), in *M. sexta* the application of OA via perfusion with the tip-recording electrode increased both the SP amplitude and initial AP frequency during the photophase. The findings in *M. sexta* differ partly from tip recordings from trichoid sensilla of *M. brassicae* (Grosmaitre et al. 2001), *Periplaneta americana* (Zhukovskaya and Kapitsky 2006), and *A. polyphemus* (Pophof 2000). These recordings only showed OA-dependent increases in the pheromone-dependent AP frequency, but no change in the SP amplitude. Possibly, these differences are caused either by differing application methods, or they are indicative for different locations of OA receptors. Alternatively, the recordings could have been performed during different ZTs not considering endogenous circadian changes in the modulated state of the ORNs.

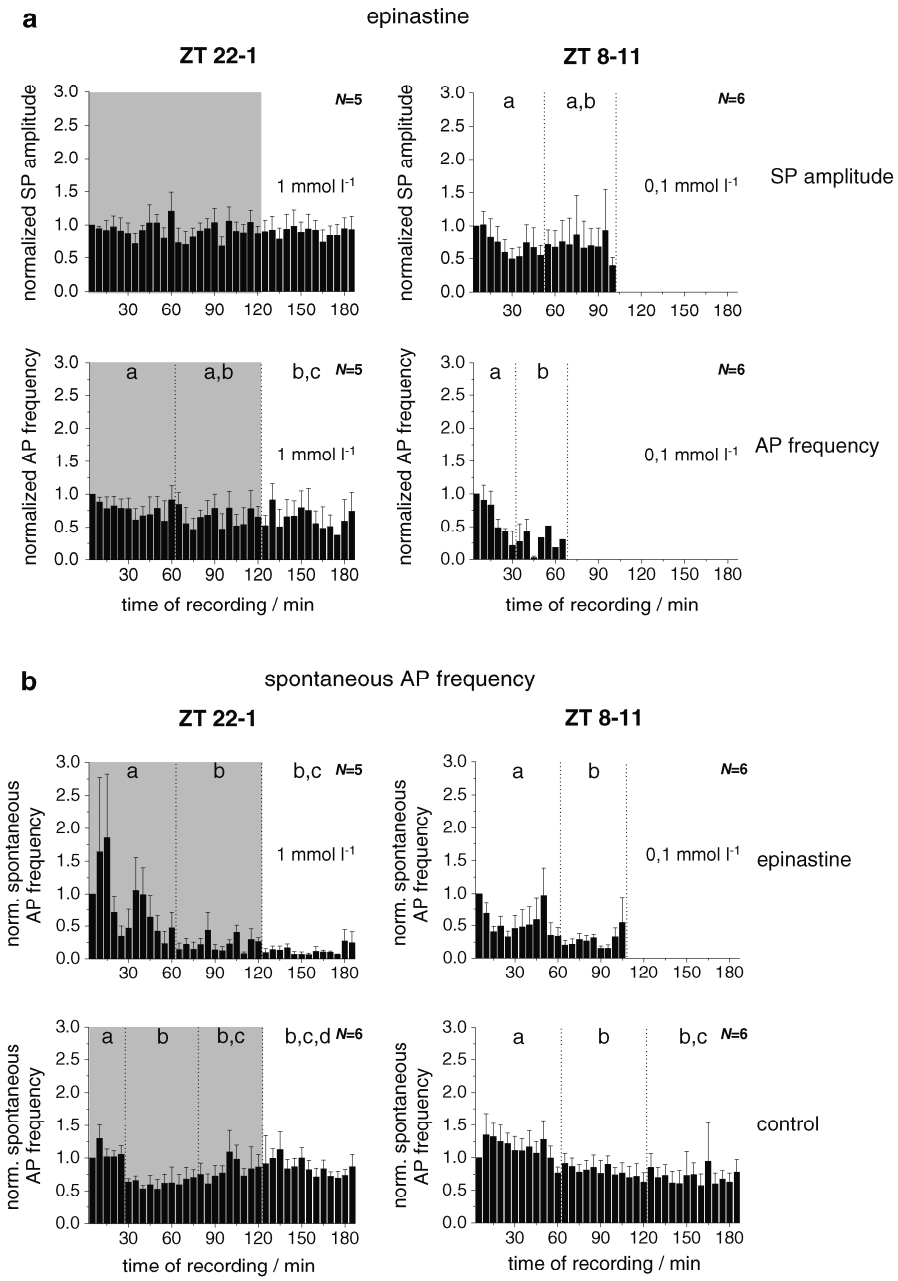


Fig. 8 Effects of the OA receptor antagonist EPI on BAL reNSsponses (**a**) and spontaneous activity of ORNs (**b**). **a** The application of 0.1 mmol l^{-1} EPI significantly decreased the BAL-dependent AP frequency in recordings at ZT 8-11 (Mann–Whitney test, $P < 0.01$), but had no significant influence on the SP amplitude. At ZT 22-1 EPI although applied with a concentration of 1 mmol l^{-1} had only a weak but significant effect on the BAL-dependent AP frequency (Mann–Whitney test, $P < 0.01$). In addition EPI (1 mmol l^{-1} at ZT 22-1; 0.1 mmol l^{-1} at ZT 8-11) decreased the normalized mean of the spontaneous AP frequency significantly at both ZTs (**b**) (Mann–Whitney test, $P < 0.001$). However, also in the controls at ZT 8-11 and ZT 22-1 significant, but in comparison to the EPI effects, much weaker dNSecreases of the mean spontaneous AP frequency were found (ANOVA and Tukey HSD post hoc test; $\alpha = 0.001$, $P < 0.001$ or Mann–Whitney test, $P < 0.001$). *Different lower case letters* denote significant differences between tested groups of mean values

Our results also support the previously stated hypothesis that OA plays a key role in the disadaptation, rather than sensitization of ORNs of different insect species (Grosmaitre et al. 2001). In accordance with this hypothesis the endogenous decrease in the mean AP frequency at ZT 8-11, which could reflect an additional adaptation mechanism, was antagonized by OA and TA application. Furthermore, OA and TA enhanced BAL detection only during the photophase when ORNs are adapted, but not during the scotophase, when ORNs are already maximally sensitized. Apparently, an OA-dependent dendritic conductance is activated during the photophase which enhances the adapted pheromone-dependent receptor potential, causing increases in the SP amplitude. The stronger depolarization might then also increase the pheromone-dependent initial AP frequency. The OA-dependent AP amplitude reduction of pheromone responses during the photophase is also most likely due to a decrease in the resistance of the ORNs. This resistance decrease might be caused by OA-dependent ion channel openings. But because EPI affects the OA-dependent changes in the pheromone-dependent SP amplitude and the pheromone-dependent AP response differently,

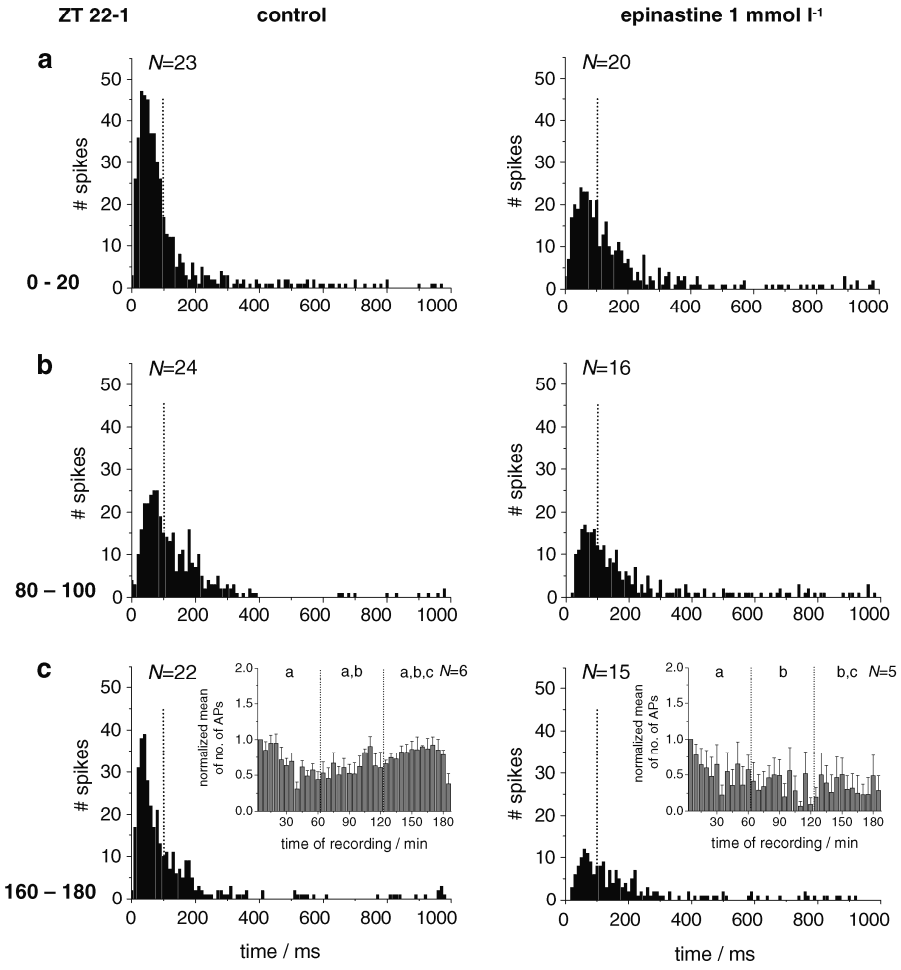


Fig. 9 The distribution of APs in response to stimulation with 10 µg BAL for recordings at ZT 22-1. PSTHs (binwidth = 10 ms) for recordings from ZT 8-11 for the beginning (**a**; 0–20 min), middle (**b**; 80–100 min) and end of the recordings (**c**; 160–180 min). The application of 1 mmol l^{-1} EPI shifted the reNSsponses from a phasic to a tonic response-characteristic. Also, the number of APs occurring during the first 100 ms dNSecreased significantly (ANOVA and Tukey HSD post hoc test; $\alpha = 0.01$, $P < 0.01$). In the controls at ZT 22-1 a temporary decrease in the APs during the first 100 ms of the response was observed, but it was not significant (Mann–Whitney test, $P < 0.01$). *Different lower case letters* denote significant differences between tested groups of mean values

more than one OA-dependent mechanism affects pheromone transduction. As previously hinted by findings of pheromone responses by non-neuronal cells of the sensillum the SP amplitude appears not only to reflect the receptor potentials of the sensory neurons. It also appears to include responses of non-neuronal cells which generate the transepithelial potential (Stengl and Zintl 1996; Stengl et al. 2001). Thus, it appears likely that pheromone and OA synergistically affect non-neuronal cells in addition to the ORNs.

Furthermore, EPI did not affect the BAL-dependent SP amplitude, but it decreased the BAL-dependent AP frequency. Thus, apparently more than one OA receptor exists in *M. sexta* antennae which might also differ in its TA-binding properties. At ZT 8-11 TA also increased the SP amplitude and the AP frequency, whereby the increase of the SP amplitude was 2.5× weaker than in recordings with application of OA. We assume that either the OA receptor that is involved in this modulation also recognizes TA or there is an additional TA receptor present which couples to the same enzyme as the OA receptor. Almost all of the invertebrate TA receptors that were cloned and pharmacologically investigated until now were found to be negatively coupled to an adenylyl cyclase (Roeder 2005; Saudou et al. 1990; Ohta et al. 2003). In contrast, the large majority of OA receptors are positively coupled to an adenylyl cyclase (Roeder 2005; Evans and Maqueira 2005; Farooqui 2007) and also show a lower affinity to TA (Han et al. 1998; Bischof and Enan 2004; Maqueira et al. 2005; Balfanz et al. 2005; Farooqui 2007). Recently OA receptors were cloned in *M. sexta and M. brassicae* and localized in antennal tissue (Dacks et al. 2006; Brigaud et al. 2009). However, despite the fact that the *M. sexta* OA receptor has not been pharmacologically characterized, it has a high sequence similarity to other insect OA receptors that were classified as OA-α-adrenergic-like receptors which increase both Ca^{2+} and cAMP levels and show a higher affinity to OA than to TA (Evans and Maqueira 2005; Farooqui 2007). Therefore, it is likely that the receptor that mediates the described effects is an OA receptor which has also a lower

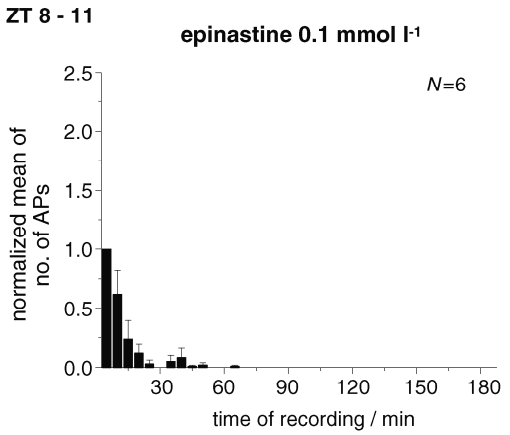


Fig. 10 The normalized mean of the number of APs occurring during the first 100 ms of the responses for EPI recordings at ZT 8-11. The application of 0.1 mmol l^{-1} EPI significantly decreased the numbers of APs within a short time. The time course of the EPI effect was compared to the respective control at ZT 8-11 (Mann–Whitney test, $P < 0.001$)

affinity to TA and possibly activates an adenylyl cyclase. The characterization of cyclic nucleotide-gated ion channels in patch clamp recordings of cultured ORNs of *M. sexta* support this assumption (Dolzer et al. 2008; Krannich and Stengl 2008).

Time dependency of biogenic amine effects

Our study provided new evidence for the involvement of peripheral ORNs in the time-dependent regulation of the sensitivity to pheromone. It was shown that the mean AP frequency in responses to stimulation was significantly lower in control recordings at ZT 8-11 than at ZT 22-1. In addition the application of OA and TA at ZT 8-11 antagonized this possible additional adaptation mechanism, by increasing the mean AP frequency back to its initial values measured at ZT 22-1. In *M. sexta* it was previously shown that OA concentrations express a circadian rhythm in the hemolymph with a maximum concentration in the scotophase when the nocturnal moths show highest flight activity (Lehman 1990) and maximal mating behavior (Lingren et al. 1977). OA concentrations were low during the photophase when nocturnal moths are inactive. Also, in other moths time-dependent variations in the concentration of biogenic amines including OA in the hemolymph were reported which were correlated with maxima of behavioral activity (Linn Jr et al. 1994, 1996). For *T. ni* circadian changes in the level of OA in the brain, ganglia and CC/CA were found and together with the pheromone dependent behavior proved to be under the control of an endogenous oscillator (Linn Jr et al. 1996). Furthermore, a time dependency in the effects after OA injection was observed. In *T. ni* it was shown that OA injections can increase the rate of pheromone source finding significantly, only when injected prior to the beginning of the scotophase and, thus, prior to the endogenous maximum of OA concentrations in the hemolymph (Linn Jr et al. 1992, 1994). Also, for *L. dispar* the OA application was only followed by an increase in the proportion of males making source contact when injected during the photophase prior to the light–dark transition (Linn Jr and Roelofs 1992). These results are in accordance with our findings, showing that the application of OA and TA was only effective during low OA levels in the photophase but not during the scotophase when OA levels in the hemolymph were endogenously elevated. Possibly in the scotophase, the high endogenous OA concentrations already maximally disadapted the ORNs and thus, further OA application is ineffective. The correlation between the decrease in OA levels and the decrease in the mean AP frequency in the photophase further supports the hypothesis that the regulation of the OA level is the key factor in the adjustment of the sensitivity of ORNs to pheromone. In addition, because EPI was hardly effective during the scotophase, an additional mechanism next to OA appeared to sensitize/disadapt ORNs in the scotophase, which might overshadow the OA effect. Possibly, this additional mechanism is a circadian change in the abundance of pheromone receptors, as shown in the fruitfly (Tanoue et al. 2008).

In *Drosophila melanogaster* it was shown that a PERIOD-based (PER) endogenous clock in the ORNs drives circadian rhythms in olfactory sensitivity, whereas electroantennogram (EAG) amplitudes and rhythmic localization of olfactory receptors were controlled via rhythms in the level of a G-protein-coupled receptor kinase (Krishnan et al. 1999, 2008; Tanoue et al. 2004, 2008; Zhou et al. 2005). In contrast, investigations searching for circadian rhythms of moths and cockroaches to pheromones showed that these rhythms were controlled at the level of the antennal lobe and midbrain (Payne et al. 1969; Worster and Seabrook 1988; Rosén et al. 2003; Page and Koelling 2003). Our results show that OA-dependent modulation of pheromone sensitivity also occurs at the periphery. In addition immunocytochemical studies in *M. sexta* found PER expressed in ORNs and their supporting cells (Schuckel et al. 2007) and PER-like and CRYPTOCHROME-like immunoreactivity was found in ORNs of *M. brassicae* (Merlin et al. 2006). Furthermore, in *Spodoptera littoralis* circadian rhythms in responses to pheromone stimulation in EAG recordings and in the expression of different clock genes in the antenna were described (Merlin et al. 2007). This supports the hypothesis that rhythms in pheromone sensitivity also occur at the periphery and an endogenous circadian pacemaker controlling these rhythms is located in the antenna or in the ORNs. But, it remains to be studied whether a PER-based circadian oscillator in *M. sexta*

antenna or ORNs controls their sensitivity to pheromone stimulation and OA via circadian expression of pheromone receptors.

Effects on the AP distribution

For moths the intermittency of the pheromone signal is critical for their upwind flight response to pheromone. In wind tunnel experiments a higher frequency of the pheromone signal increased the source contacts of male *Heliothis virescens* (Vickers and Baker 1992) and the structure of the pheromone plume had a stronger impact on the flight pattern than a 1,000-fold increase in the pheromone dosage (Mafra-Neto and Cardé 1995). Interestingly, in *A. polyphemus* a temperature decrease rendered pheromone responses of ORNs more tonic and the cooled cells lost their ability to encode pulsed pheromone signals (Kodadová 1996). Also, in *Helicoverpa zea* ORNs with a phasic response pattern were able to encode higher frequencies of pulsed pheromone signals than ORNs with a tonic response characteristic (Almaas et al. 1991). Thus, a more phasic and less tonic response pattern of ORNs improves their temporal resolution of pheromone pulses.

We showed that both biogenic amines when applied at ZT 8-11 render the pheromone responses more phasic in comparison to the control recordings. Furthermore, control recordings at ZT 22-1 and ZT 1-4 (Flecke et al. 2006) showed that during the late scotophase and early photophase when endogenous OA concentrations are high pheromone responses are more phasic. In addition, the OA receptor antagonist EPI rendered pheromone responses more tonic at ZT 22-1. Therefore, our experiments suggested for the first time that the stress hormone OA could be necessary for the resolution of pheromone pulses during the scotophase and the photophase. We previously demonstrated that in control recordings at ZT 8-11 the pheromone responses of stimulated ORNs get increasingly tonic with recording duration. The time-dependent shift to tonic responses was enhanced by the application of 8bcGMP but only at ZT 8-11 (Flecke et al. 2006). Thus, during the photophase the decline in the endogenous OA concentration and an additional possibly clock-controlled cGMP-dependent mechanism renders the moth unresponsive to pheromone pulses. While in the scotophase endogenous OA levels are high enabling the males to detect pheromone pulses as phasic responses in control recordings confirm (Lehman 1990; Flecke et al. 2006). Therefore, we hypothesize that OA is a necessary prerequisite for high temporal resolution of pheromone pulses during all ZTs. Since we did not test whether EPI is solely affecting OA receptors and no other biogenic amine or peptide receptors in *M. sexta* we do not know whether OA alone is a prerequisite for pheromone pulse detection.

OA and EPI affect the spontaneous AP frequency of ORNs

Spontaneous bursting of ORNs was reported for several mammals as well as for invertebrates and, thus, appears to be an endogenous property of ORNs (Frings and Lindemann 1988; Reisert and Matthews 2001; Holy et al. 2000; Dolzer et al. 2001; Bobkov and Ache 2007). In addition, it was suggested that spontaneous bursting enhances the detection of weak intermittent odor signals (Bobkov and Ache 2007). Because the application of OA increased the spontaneous AP frequency by decreasing the interburst-intervals and by prolonging the bursts during the photophase and EPI strongly decreased the spontaneous activity both in recordings at ZT 22-1 and ZT 8-11, OA appears to be a necessary prerequisite for high spontaneous activity during photophase and scotophase. Also, in the tangential abdominal ganglion of *L. dispar* the application of OA increased the spontaneous burst frequency and reduced the interburst-intervals of interneurons (Olianas et al. 2005). The application of the OA-antagonist mianserin reduced the mean spike frequency of spontaneous activity and abolished spontaneous bursts completely. Thus, the effect of OA on the burst-behavior of different neurons seems to be a general characteristic of this biogenic amine. Our results are in accordance with findings from Grosmaitre et al. (2001) who observed an increase in the spontaneous AP frequency after OA application in tip recordings of unstimulated trichoid sensilla of *M. brassicae.* However, for *A. polyphemus* an OA-dependent increase in the spontaneous AP frequency was only observed during continuous low level pheromone stimulation (Pophof 2000). Also, Dolzer et al. (2001) did not observe any effect of OA on the spontaneous AP frequency of unstimulated ORNs of *M. sexta*. In our experiments the spontaneous activity was recorded between pheromone stimuli and the ORNs were temporarily adapted during the first minutes after the BAL stimuli and also long term adapted as the decrease of the mean spontaneous AP frequency in controls at ZT 22-1 and ZT 8-11 showed. Thus our results partly support the hypothesis of Grosmaitre et al (2001) that OA rather disadapt than sensitize ORNs. It is also likely that pheromone and OA have synergistic effects on the spontaneous activity.

In *Drosophila*, it was shown that the olfactory receptor heterodimer composed of OR47a and OR83b forms a leaky ion channel which causes steady influx of cations into the sensory neuron, thus affecting the spontaneous activity of the cells (Sato et al. 2008). Interestingly, Wicher et al. (2008) showed that the Or83b receptor is activated cAMP dependently. Current experiments will examine our hypothesis whether in *M. sexta* OA increases cAMP levels in the antenna and if OA-dependent cAMP levels control the activity of the pheromone receptor ion channel complex affecting pheromone sensitivity as well as temporal resolution of pheromone pulses.

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References

Almaas TJ, Christensen TA, Mustaparta H (1991) Chemical communication in heliothine moths: I. Antennal receptor neurons encode several features of intra- and interspecific odorants in the male corn earworm moth Helicoverpa zea. J Comp Physiol A 169:249–258

Balfanz S, Strünker T, Frings S, Baumann A (2005) A family of octopamine [corrected] receptors that specifically induce cyclic AMP production or Ca^{2+} release in *Drosophila melanogaster*. J Neurochem 93:440–451 (Erratum: J Neurochem 94:1168, 2005)

Bell RA, Joachim FA (1976) Techniques for rearing laboratory colonies of tobacco hornworms and pink bollworms. Ann Entomol Soc Am 69:365–373

Bischof LJ, Enan EE (2004) Cloning, expression and functional analysis of an octopamine receptor from *Periplaneta americana*. Insect Biochem Mol Biol 34:511–521

Bobkov YV, Ache BW (2007) Intrinsically bursting olfactory receptor neurons. J Neurophysiol 97:1052–1057

Brigaud I, Grosmaître X, François MC, Jacquin-Joly E (2009) Cloning and expression pattern of a putative octopamine/tyramine receptor in antennae of the noctuid moth *Mamestra brassicae*. Cell Tissue Res 335:445–463

Dacks AM, Dacks JB, Christensen TA, Nighorn AJ (2006) The cloning of one putative octopamine receptor and two putative serotonin receptors from the tobacco hawkmoth *Manduca sexta*. Insect Biochem Mol Biol 36:741–747

Dolzer J (2002) Mechanisms of modulation and adaptation in pheromone-sensitive trichoid sensilla of the hawkmoth *Manduca sexta*. Ph.D. thesis, Philipps-Universität, Marburg, http://archiv.ub.uni-marburg.de/diss/z2002/0185

Dolzer J, Krannich S, Fischer K, Stengl M (2001) Oscillations of the transepithelial potential of moth olfactory sensilla are influenced by octopamine and serotonin. J Exp Biol 204:2781–2794

Dolzer J, Fischer K, Stengl M (2003) Adaptation in pheromone-sensitive trichoid sensilla of the hawkmoth *Manduca sexta*. J Exp Biol 206:1575–1588

Dolzer J, Krannich S, Stengl M (2008) Pharmacological investigation of protein kinase C- and cGMP-dependent ion channels in cultured olfactory receptor neurons of the hawkmoth *Manduca sexta*. Chem Senses 33:803–813

Evans PD, Maqueira B (2005) Insect octopamine receptors: a new classification scheme based on studies of cloned *Drosophila* G-protein coupled receptors. Invert Neurosci 5:111–118

Farooqui T (2007) Octopamine-mediated neuromodulation of insect senses. Neurochem Res 32:1511–1529

Flecke C, Dolzer J, Krannich S, Stengl M (2006) Perfusion with cGMP analogue adapts the action potential response of pheromone-sensitive sensilla trichoidea of the hawkmoth *Manduca sexta* in a daytime-dependent manner. J Exp Biol 209:3898–3912

Frings S, Lindemann B (1988) Odorant response of isolated olfactory receptor cells is blocked by amiloride. J Membr Biol 105:233–243

Grosmaitre X, Marion-Poll F, Renou M (2001) Biogenic amines modulate olfactory receptor neurons firing activity in *Mamestra brassicae*. Chem Senses 26:653–661

Han KA, Millar NS, Davis RL (1998) A novel octopamine receptor with preferential expression in *Drosophila* mushroom bodies. J Neurosci 18:3650–3658

Holy TE, Dulac C, Meister M (2000) Responses of vomeronasal neurons to natural stimuli. Science 289:1569–1572

Itagaki H, Conner WE (1988) Calling behavior of *Manduca sexta* (L.) (Lepidoptera: Sphingidae) with notes on the morphology of the female sex pheromone gland. Ann Entomol Soc Am 81:798–807

Kaissling KE, Hildebrand JG, Tumlinson JH (1989) Pheromone receptor cells in the male moth *Manduca sexta*. Arch Insect Biochem Physiol 10:273–279

Keil TA (1989) Fine structure of the pheromone-sensitive sensilla on the antenna of the hawkmoth, *Manduca sexta*. Tissue Cell 21:139–151

Kodadová B (1996) Resolution of pheromone pulses in receptor cells of *Antheraea polyphemus* at different temperatures. J Comp Physiol A 179:301–310

Krannich S, Stengl M (2008) Cyclic nucleotide-activated currents in cultured olfactory receptor neurons of the hawkmoth *Manduca sexta*. J Neurophysiol 100:2866–2877

Krishnan B, Dryer SE, Hardin PE (1999) Circadian rhythms in olfactory responses of *Drosophila melanogaster*. Nature 400:375–378

Krishnan P, Chatterjee A, Tanoue S, Hardin PE (2008) Spike amplitude of single-unit responses in antennal sensillae is controlled by the *Drosophila* circadian clock. Curr Biol 18:803–807

Lehman HK (1990) Circadian control of *Manduca sexta* flight. Abstr Soc Neurosci 16:1334

Lee JK, Strausfeld NJ (1990) Structure, distribution, and number of surface sensilla and their receptor cells on the antennal flagellum of the male sphinx moth *Manduca sexta*. J Neurocytol 19:519–538

Lingren PD, Greene GL, Davis DR, Baumhover AH, Henneberry TJ (1977) Nocturnal behavior of four lepidopteran pests that attack tobacco and other crops. Ann Entomol Soc Am 70:161–167

Linn CE Jr, Roelofs WL (1984) Sublethal effects of neuroactive compounds on pheromone response thresholds in male oriental fruit moths. Arch Insect Biochem Physiol 1:331–344

Linn CE Jr, Roelofs WL (1986) Modulatory effects of octopamine and serotonin on male sensitivity and periodicity of response to sex pheromone in the cabbage looper moth, *Trichoplusia ni*. Arch Insect Biochem Physiol 3:161–171

Linn CE Jr, Roelofs WL (1992) Role of photoperiod cues in regulating the modulatory action of octopamine on pheromone-response thresholds in the cabbage looper moth. Arch Insect Biochem Physiol 20:285–302

Linn CE Jr, Campbell MG, Roelofs WL (1992) Photoperiod cues and the modulatory action of octopamine and 5-hydroxytryptamine on locomotor and pheromone response in male gypsy moths, *Lymantria dispar*. Arch Insect Biochem Physiol 20:265–284

Linn CE Jr, Poole KR, Roelofs WL (1994) Studies on biogenic amines and their metabolites in nervous tissue and hemolymph of adult male cabbage looper moths I. Quantitation of photoperiod changes. Comp Biochem Physiol 108:73–85

Linn CE Jr, Campbell MG, Poole KR, Wu W-Q, Roelofs WL (1996) Effects of photoperiod on the circadian timing of pheromone response in male *Trichoplusia ni*: relationship to the modulatory action of octopamine. J Insect Physiol 42:881–891

Mafra-Neto A, Cardé RT (1995) Influence of plume structure and pheromone concentration on upwind flight of *Cadra cautella* males. Physiol Entomol 20:117–133

Maqueira B, Chatwin H, Evans PD (2005) Identification and characterization of a novel family of *Drosophila* beta-adrenergic-like octopamine G-protein coupled receptors. J Neurochem 94:547–560

Merlin C, François MC, Queguiner I, Maibèche-Coisné M, Jacquin-Joly E (2006) Evidence for a putative antennal clock in *Mamestra brassicae*: molecular cloning and characterization of two clock

genes—*period and cryptochrome*—in antennae. Insect Mol Biol 15:137–145

Merlin C, Lucas P, Rochat D, François MC, Maibèche-Coisne M, Jacquin-Joly E (2007) An antennal circadian clock and circadian rhythms in peripheral pheromone reception in the Moth *Spodoptera littoralis*. J Biol Rhythms 22:502–514

Ohta H, Utsumi T, Ozoe Y (2003) B96Bom encodes a *Bombyx mori* tyramine receptor negatively coupled to adenylate cyclase. Insect Mol Biol 12:217–223

Olianas MC, Solari P, Garau L, Liscia A, Crnjar R, Onali P (2005) Stimulation of cyclic AMP formation and nerve electrical activity by octopamine in the terminal abdominal ganglion of the female gypsy moth *Lymantria dispar*. Brain Res 1071:63–74

Page TL, Koelling E (2003) Circadian rhythm in olfactory response in the antennae controlled by the optic lobe in the cockroach. J Insect Physiol 49:697–707

Payne TL, Shorey HH, Gaston LK (1969) Sex pheromones of noctuid moths: factors influencing antennal responsiveness in males of *Trichoplusia ni*. J Insect Physiol 16:1043–1055

Pittendrigh CS, Eichhorn JH, Minis DH, Bruce VG (1970) Circadian systems, VI. Photoperiodic time measurement in *Pectinophora gossypiella*. Proc Natl Acad Sci USA 66:758–764

Pophof B (2000) Octopamine modulates the sensitivity of silkmoth pheromone receptor neurons. J Comp Physiol A 186:307–313

Pophof B (2002) Octopamine enhances moth olfactory responses to pheromones, but not those to general odorants. J Comp Physiol A 188:659–662

Reisert J, Matthews HR (2001) Responses to prolonged odour stimulation in frog olfactory receptor cells. J Physiol 534:179–191

Roeder T (2005) Tyramine and octopamine: ruling behavior and metabolism. Annu Rev Entomol 50:447–477

Roeder T, Degen J, Gewecke M (1998) Epinastine, a highly specific antagonist of insect neuronal octopamine receptors. Eur J Pharmacol 349:171–177

Rosén WQ (2002) Endogenous control of circadian rhythms of pheromone production in the turnip moth *Agrotis segetum*. Arch Insect Biochem Physiol 50:21–30

Rosén WQ, Han GB, Löfstedt C (2003) The circadian rhythm of the sex-pheromone-mediated behavioral response in the turnip moth, *Agrotis segetum*, is not controlled at the peripheral level. J Biol Rhythms 18:402–408

Rospars JP, Lánský P, Vaillant J, Duchamp-Viret P, Duchamp A (1994) Spontaneous activity of first- and second-order neurons in the frog olfactory system. Brain Res 662:31–44

Sanes JR, Hildebrand JG (1976) Origin and morphogenesis of sensory neurons in an insect antenna. Dev Biol 51:300–319

Sasaki M, Riddiford LM (1984) Regulation of reproductive behaviour and egg maturation in the tobacco hawk moth, *Manduca sexta*. Physiol Entomol 9:315–327

Sato K, Pellegrino M, Nakagawa T, Nakagawa T, Vosshall LB, Touhara K (2008) Insect olfactory receptors are heteromeric ligand-gated ion channels. Nature 452:1002–1006

Saudou F, Amlaiky N, Plassat J-L, Borelli E, Hen R (1990) Cloning and characterization of a *Drosophila* tyramine receptor. EMBO J 9:3611–3617

Schuckel J, Siwicki KK, Stengl M (2007) Putative circadian pacemaker cells in the antenna of the hawkmoth *Manduca sexta*. Cell Tissue Res 330:271–278

Schröter U, Malun D, Menzel R (2007) Innervation pattern of suboesophageal ventral unpaired median neurones in the honeybee brain. Cell Tissue Res 327:647–667

Silvegren G, Löfstedt C, Rosén WQ (2005) Circadian mating activity and effect of pheromone pre-exposure on pheromone response rhythms in the moth *Spodoptera littoralis*. J Insect Physiol 51:277–286

Stengl M, Zintl R (1996) NADPH-diaphorase staining in the antenna of the moth *Manduca sexta*. J Exp Biol 199:1063–1072

Stengl M, Zintl R, de Vente J, Nighorn A (2001) Localization of cGMP-immunoreactivity and of soluble guanylyl cyclase in antennal sensilla of the hawkmoth *Manduca sexta*. Cell Tissue Res 304:409–421

Tanoue S, Krishnan T, Krishnan B, Dryer SE, Hardin PE (2004) Circadian clocks in antennal neurons are necessary and sufficient for olfaction rhythms in *Drosophila*. Curr Biol 14:638–649

Tanoue S, Krishnan P, Chatterjee A, Hardin PE (2008) G protein-coupled receptor kinase 2 is required for rhythmic olfactory responses in *Drosophila*. Curr Biol 18:787–794

Tumlinson JH, Brennan MM, Doolittle RE, Mitchell ER, Brabham A, Mazomemos BE, Baumhover AH, Jackson DM (1989) Identification of a pheromone blend attractive to *Manduca sexta* (L.) males in a wind tunnel. Arch Insect Biochem Physiol 10:255–271

Vickers NJ, Baker TC (1992) Male *Heliothis virescens* maintain upwind flight in response to experimentally pulsed filaments of their sex pheromone *(Lepidoptera: Noctuidae)*. J Insect Behav 5:669–687

Wicher D, Schäfer R, Bauernfeind R, Stensmyr MC, Heller R, Heinemann SH, Hansson BS (2008) *Drosophila* odorant receptors are both ligand-gated and cyclic-nucleotide-activated cation channels. Nature 452:1007–1011

Worster AS, Seabrook WD (1988) Electrophysiological investigation of diel variations in the antennal sensitivity of the male spruce budworm moth *Choristoneura fumiferana* (Lepidoptera: Tortricidae). J Insect Physiol 35:1–5

Zhou X, Yuan C, Guo A (2005) *Drosophila* olfactory response rhythms require clock genes but not pigment dispersing factor or lateral neurons. J Biol Rhythms 20:237–244

Zhukovskaya MI, Kapitsky SV (2006) Activity modulation in cockroach sensillum: the role of octopamine. J Insect Physiol 52:76–86

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Perfusion with cAMP analogue affects pheromone-sensitive trichoid sensilla of the hawkmoth *Manduca sexta* in a time-dependent manner

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Abstract
Octopamine disadapts pheromone-sensitive olfactory receptor neurons of *Manduca sexta* time-dependently. Because the majority of insect octopamine receptors are positively coupled to adenylyl cyclases we examined whether cyclic adenosine monophosphate (cAMP) mimics octopamine-dependent modulation of pheromone transduction in a time-dependent manner. Long-term tip recordings of single trichoid sensilla of *Manduca sexta* were performed during three Zeitgebertimes (ZTs, ZT 0 = lights on), while stimulating the sensilla with the main pheromone component bombykal in a non-adapting protocol. The membrane-permeable cAMP analogue 8bcAMP increased the normalized sensillar potential amplitude in recordings at ZT 1-4 and ZT 8-11 when olfactory receptor neurons (ORNs) were adapted but not at ZT 22-1, when ORNs were sensitized. In addition in recordings with 10 µg bombykal stimulation 8bcAMP antagonized an endogenous decrease in the mean sensillar potential amplitude at ZT 1-4 and ZT 8-11. In contrast to octopamine, 8bcAMP did not affect the normalized initial action potential frequency, the distribution of action potentials in responses or the shift to lower mean action potential frequencies at ZT 8-11. Furthermore, 8bcAMP increased the spontaneous action potential frequency in recordings with 1 µg bombykal stimulation at ZT 8-11 and antagonized an endogenous decrease in the mean spontaneous action potential frequency at ZT 1-4 and ZT 8-11. However, the 8bcAMP-dependent effect on the spontaneous activity was weaker than the octopamine effect and 8bcAMP did not predominantly affect the burst behaviour of the ORNs. In conclusion, our results show that cAMP only partly mimics the octopamine-dependent disadaptation of olfactory receptor neurons during the photophase.

Keywords: insect olfaction**,** pheromone transduction, cyclic AMP, octopamine, time-dependent differences

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Abbreviations

8bcAMP	8-bromo 3´,5´-cyclic adenosine monophosphate
AP	Action potential
BAL	Bombykal
cAMP	3',5'-cyclic adenosine monophosphate
EAG	Electroantennogram
IP_3	Inositol-1,4,5- triphosphate
HCN	Hypolarization-activated cyclic nucleotide-gated
ISI	Interspike interval
OA	Octopamine
OR	Olfactory receptors
ORN	Olfactory receptor neuron
PSTH	Post-stimulus-time-histogram
SP	Sensillar potential
TEP	Transepithelial potential
ZT	Zeitgebertime

Introduction

The second messenger 3',5'-cyclic adenosine monophosphate (cAMP) is involved in both invertebrate and vertebrate olfaction. Whereas cAMP signalling is best understood in olfactory receptor neurons (ORNs) of vertebrates, less is known about its function in invertebrate olfaction. In vertebrate ORNs of the main olfactory epithelium olfactory receptors activate G-proteins odour-dependently, which then stimulate adenylyl cyclases (Nakamura 2000). In invertebrate ORNs phospholipase C-dependent transduction cascades are activated after odour stimulation (Stengl et al., 1999; Ache and Young, 2005). Only few studies investigated the role of cAMP in invertebrate olfaction. In lobster ORNs the cAMP pathway leads to hyperpolarization and codes inhibitory responses (Hatt and Ache, 1994; Boekhoff et al., 1994; Doolin and Ache, 2005). In antennae of *Antheraea pernyi* the perfusion with cAMP increased electroantennogram (EAG) amplitudes in responses to stimulation with pheromone (Villet, 1978). In addition, overexpression of the cAMP-phosphodiesterase encoding *dunce* gene in olfactory organs of *Drosophila melanogaster* affected both the behaviour in y-maze experiments (Gomez-Diaz et al., 2004) and EAGs recorded from the antennae (Martín et al., 2001). Recently

it was shown that heterologously expressed insect olfactory receptors (ORs) form ligand-gated ion channel complexes, with highly conserved chaperone proteins, such as OR83b (Sato et al., 2008; Wicher et al., 2008). In addition, the stimulation of OR22a was followed by the activation of an adenylyl cyclase. This resulted in the binding of cAMP to OR83b, thereby activating a slower, but stronger additional receptor current (Wicher et al., 2008). This cAMP-dependent mechanism was suggested to augment weak odour signals.

Several studies revealed that diurnal production, release of pheromones and calling behaviour of female moths are controlled by unknown circadian pacemakers (Baker and Cardé, 1979; Choi et al., 1998; Rosén, 2002). Also male moths express circadian rhythms in their responsiveness to pheromone with maxima in the scotophase (Linn Jr et al., 1992; Linn Jr et al., 1996; Rosén et al., 2003). For *Manduca sexta* it was shown that the maxima in calling behaviour of females (Itagaki and Conner, 1988) correlated with maximal male flight activity in the scotophase (Lingren et al., 1977; Sasaki and Riddiford, 1984). Furthermore, ORNs of *M. sexta* adapted with beginning of the photophase and responded pheromone-dependently with lower mean action potential (AP) frequencies at the middle of photophase (Flecke and Stengl, 2009).

The biogenic amine octopamine (OA) was shown to modulate the sensitivity of pheromone-sensitive ORNs. Injections of OA into the hemolymph enhanced the responsiveness of male moths to pheromone in behavioural experiments (Linn Jr and Roelofs, 1986; Linn Jr and Roelofs, 1992; Linn Jr et al., 1992) and sensitized ORNs of different moth species during tip recordings of pheromone-sensitive trichoid sensilla (Grosmaitre et al., 2001; Pophof, 2000; Pophof, 2002). In addition, it was shown that the OA effects are time-dependent and that OA disadapts ORNs of *M. sexta* during the middle of the photophase, suggesting a circadian control of OA action (Flecke and Stengl, 2009). The assumption that the OA-dependent modulation is controlled by a circadian clock is further supported by circadian changes of OA concentrations in the hemolymph and brain, correlating with circadian rhythms in mating behaviour (Linn Jr et al., 1994; Linn Jr et al., 1996; Lehman, 1990). Since OA receptors are often positively coupled to adenylyl cyclases (Balfanz et al., 2005; Evans and Maqueira, 2005; Farooqui, 2007), it is likely that the OA effects are mediated by increases in the cAMP concentration. The cyclic nucleotide sensitive binding site on OR83b is a possible target for OA-dependent modulation of cAMP levels. In addition, cyclic nucleotide sensitive channels were characterized in patch clamp recordings of cultured ORNs of *M. sexta* (Dolzer et al., 2008; Krannich and Stengl, 2008).

To determine whether cAMP is involved in the primary signal transduction process of pheromone-sensitive trichoid sensilla of *M. sexta* and to elucidate the role of cAMP in the time-dependent modulation of the sensitivity to pheromone, long-term tip recordings were performed during three different Zeitgebertimes (ZTs: ZT 22-1, ZT 1-4 and ZT 8-11). The membrane-permeable cAMP analogue 8-bromo 3´,5´-cyclic adenosine monophosphate (8bcAMP) was applied via perfusion over the recording electrode with beginning of the recordings. The trichoid sensilla were stimulated with the main pheromone component bombykal (BAL) in a non-adapting stimulation protocol (BAL dose 1 or 10 µg, interstimulus-interval 5 min). Several time-dependent and also pheromone-dose-dependent effects of 8bcAMP on the sensillar potential (SP) and on the spontaneous AP activity were detected.

Material and methods

Animals and preparation

In the experiments adult male *M. sexta* were used from the breeding facility at our laboratory at the University of Marburg. *M. sexta* were raised from eggs and the larvae were fed on an artificial diet (modified after Bell and Joachim, 1976). The animals were kept under long-day photoperiod conditions (L:D 17:7h) at 24-27°C and 40-60 % relative humidity. The preparation, the recording conditions and the digitization were described previously (Flecke et al., 2006). Light sources with an emission frequency above 600 nm do not function as a Zeitgeber (Pittendrigh et al., 1970). To avoid light-dependent phase shifts, red light emitting LEDs with a narrow frequency spectrum were used for the illumination of the preparation and the setup during the scotophase (Kingbright LKR 530100, Kingbright Electronics, Issum, Germany). For experiments in the scotophase animals were taken out of the rearing and handled under red light conditions till the lights were switched on at ZT 0, which is the transition from scoto- to photophase. The experiments were performed either from ZT 22-1, ZT 1-4 or ZT 8-11.

Application of 8bcAMP and pheromone stimulation

The second messenger 8-bromo adenosine 3´,5´-cyclic monophosphate (8bcAMP) with a concentration of 10 mmol l^{-1} was applied by perfusion over the recording electrode as previously described (Flecke et al., 2006). Therefore, altered sensillum lymph ringer solutions were employed. The application of 8bcAMP started with the establishment of the recordings. The rather high 8bcAMP concentration was selected to obtain significant perfusion during the first hour of the recording and is equal to the concentration of 8-bromo 3´,5´-cyclic guanosine monophosphate employed in an earlier study (Flecke et al., 2006). In addition in a subset of recordings the biogenic amine OA in a concentration of 1 mmol l^{-1} was applied in the same way. The ringer solutions were prepared with N-(2-hydroxyethyl)-piperazine-N'-(2-ethane sulfonic acid) (HEPES; all chemicals from Sigma, Deisenhofen,

Germany). The pH of the sensillum lymph and hemolymph ringer was adjusted to 6.5. The osmolality was adjusted to 475 mosmol l^{-1} for sensillum lymph ringer and to 450 mosmol l^{-1} for the hemolymph ringer using mannitol. The details of the pheromone stimulation were described previously (Flecke et al., 2006). Stimuli with doses of 10 or 1 µg of synthetic bombykal (BAL, (*E,Z*)-10,12-hexadecadienal), generously provided by T. Christensen (Tucson, AZ, USA) and J. Krieger (Stuttgart, Germany), were applied in a non-adapting stimulation protocol (stimulus duration: 50 ms) every 5 min for 180 min of the recording duration.

Acquisition protocols

In *M. sexta* each sensillum trichodeum houses two ORNs. Only one of them is responsive to the main pheromone component BAL. The BAL-sensitive ORNs generate APs with larger peak-to-peak amplitudes, which allow the discrimination of APs of both ORNs (Dolzer et al., 2001). For the digitization of the signal during pheromone responses and during spontaneous activity of the ORNs different protocols were used. The BAL responses were recorded in sweeps of approx. 5 s duration at a continuous sampling rate of 20 kHz (Clampex 8, Episodic Stimulation Mode) and the spontaneous activity between stimulations was recorded in intervals of approx. 5 min with a sampling frequency of 19.6 kHz (Clampex 8, fixed-length events).

Data analysis

The pheromone responses and the spontaneous activity were evaluated with Clampfit 8 and the Microsoft Excel Add-in XtraCell (Dolzer, 2002). All analyses were performed using the direct-current-coupled signal. To evaluate the SP, the responses were lowpass-filtered at a cutoff frequency of 50 Hz (Clampfit, Gaussian filter). Afterwards different parameters of the SP response, including the maximal SP amplitude, were evaluated as previously described (Flecke et al., 2006). Preceding to the analysis of the APs the responses were pseudo-high pass filtered as described before (Flecke et al., 2006). The main parameter AP frequency was calculated over the first 5 interspike intervals (ISI). Due to the high variability between the recordings the time courses were each normalized to the first value and then binned to 5 min intervals. For the statistical comparison of the normalized and binned time courses of 8bcAMP and control recordings of each time slot the Mann-Whitney-Test or the Student´s *t*-test were employed, depending on the distribution of the data. To analyze changes in the time course of single parameters the time courses were divided into three or four intervals and then analyzed with an Oneway ANOVA followed by the Tukey HSD post-hoc-test or with the Mann-Whitney-Test. Furthermore the mean SP amplitudes and mean AP frequencies computed over all responses recorded at each ZT were evaluated. To compare different ZTs of 8bcAMP, OA or control recordings, the complete data set for each ZT was compared to other ZTs using the Mann-Whitney-Test. For the evaluation of the distribution of APs in responses post-stimulus-time-histograms (PSTHs) (bin length 10 ms, t=0 is the start of the SP) for all recordings at one ZT were created for three intervals of the recording duration (0-20 min, 80-100 min and 160-180 min of the recording). PSTHs of 8bcAMP and control recordings from different ZTs were compared with the Mann-Whitney-Test. In addition the APs occurring in the first 100 ms of the responses were evaluated and plotted for the recording duration of 180 min. Subsequently changes in this parameter were statistically analyzed by dividing it into three intervals followed by an Oneway ANOVA with the Tukey HSD post-hoc-test or with the Mann-Whitney-Test.

Analysis of spontaneous APs and bursts

In addition, the spontaneous AP activity recorded between the stimulations was evaluated. APs with different peak-to-peak amplitudes had to be sorted. Spike sorting of spontaneous APs and calculation of bursts was performed as described before (Dolzer et al., 2001; Flecke and Stengl, 2009). The time courses of the spontaneous AP frequency, the percentage of APs in bursts, the average APs per burst, the number of bursts per bin and the number of spikes per bin were normalized to the first value and then binned to 5 min intervals. The data of control recordings was compared to recordings with 8bcAMP application by using the Mann-Whitney-Test or the Student´s *t*-test. Changes in each time course were analyzed with an Oneway ANOVA followed by the Tukey HSD post-hoc-test or with the Mann-Whitney-Test, depending on the distribution of the data. Furthermore, the mean spontaneous AP frequency, the mean number of bursts per bin and the mean number of spikes per bin calculated over all responses during each ZT were analyzed. This data was not normalized, representing absolute values. For the statistical comparison the whole data set for each ZT was compared to other ZTs using the Mann-Whitney-Test. All statistical calculations were done with SPSS (version 11) (SPSS Inc., Chicago, IL, USA).

Results

In long-term tip recordings of single pheromone-sensitive trichoid sensilla of the hawkmoth *M. sexta* it was examined whether the application of the cAMP analogue 8bcAMP mimics OA-dependent modulation of the pheromone transduction. To elucidate time-dependent differences in the effects, the experiments were performed at three different ZTs (ZT 22-1, ZT 1-4 and ZT 8-11). During the recordings with durations of three hours the sensillar lymph was perfused with 10 mmol l^{-1} 8bcAMP and the trichoid sensilla were stimulated with the main pheromone component BAL in a non-adapting stimulation protocol (BAL dose 1 or 10

µg, interstimulus-interval 5 min). Different parameters of the SP and AP response were evaluated. For a detailed explanation of the analyzed parameters see Flecke and Stengl (2006, 2009).

Perfusion with 8bcAMP increased the SP amplitude time- and BAL-dose-dependently

When comparing responses to 1 µg BAL stimulation from the beginning and end of a single recording at ZT 1-4 (Fig. 1) it was observed that the perfusion with 8bcAMP increased the SP amplitude without affecting the initial AP frequency. The augmentation of the AP amplitude in the response at the end of the recording duration was not 8bcAMP-dependent.

To determine whether this was true for all recordings, the normalized and binned SP amplitude and initial AP frequency of control and 8bcAMP recordings for the three different ZTs were evaluated. In control recordings with 10 µg BAL stimuli the SP amplitude and the initial AP frequency remained stable over the recording duration at ZT 22-1, ZT 1-4 and ZT 8-11 (Fig. 2A,B). Also for control recordings with 1 µg BAL stimuli the SP amplitude and AP frequency were not affected at ZT 22-1 and ZT 1-4 (Fig. 3A,B). Only in the controls at ZT 8-11 the AP frequency decreased slightly but significantly (Fig. 3B) (ANOVA and Tukey HSD post-hoc test; α=0.01, P<0.01). In recordings with both 10 and 1 µg BAL stimulation the application of 8bcAMP increased the SP amplitude significantly at ZT 1-4 and ZT 8-11 but not at ZT 22-1 (Figs. 2A, 3A). The 8bcAMP-dependent increases were significant when compared to the respective controls (Student's t-test for independent samples, P<0.001 or Mann-Whitney-Test, P<0.001) and in the analysis of differences in the intervals of each affected time course (ANOVA and Tukey HSD post-hoc test; α=0.001, P<0.001 or Mann-Whitney-Test, P<0.01). When comparing the increases in recordings with stimulation with 10 µg BAL at ZT 1-4 and ZT 8-11 (Fig. 2A), it was observed that the increase at ZT 1-4 was 1.3-fold stronger. However, in recordings with 1 µg BAL stimulation no significant differences in the 8bcAMP-dependent effects between ZT 1-4 and ZT 8-11 were found (Fig. 3A). In contrast, the application of 8bcAMP did not increase the normalized initial AP frequency in recordings with both BAL doses and during any ZT (ANOVA and Tukey HSD post-hoc test; α=0.01, P>0.01 or Mann-Whitney-Test, P>0.01) (Figs. 2B, 3B). However, in contrast to the control with 1 µg BAL stimulation at ZT 8-11, during 8bcAMP application the normalized AP frequency was not decreased (Student´s t-test for independent samples, P<0.001). Furthermore no 8bcAMP-dependent effects on the transepithelial potential (TEP) or on the resistance of the preparation were found that correlated with increases of the SP amplitude (data not shown). Besides no 8bcAMP-dependent effects on the AP amplitude reduction during pheromone responses were observed (data not shown).

To determine the absolute 8bcAMP effects, the not-normalized mean SP amplitude (Fig. 4A, Tab. 1) and the mean AP frequency (Fig. 4B, Tab. 2) were calculated over the absolute values of all responses at each ZT. In the control recordings with 10 µg BAL stimulation significant decreases in the mean SP amplitude were observed at ZT 1-4 and ZT 8-11 (Fig. 4A) (Mann-Whitney-Test, P<0.001).

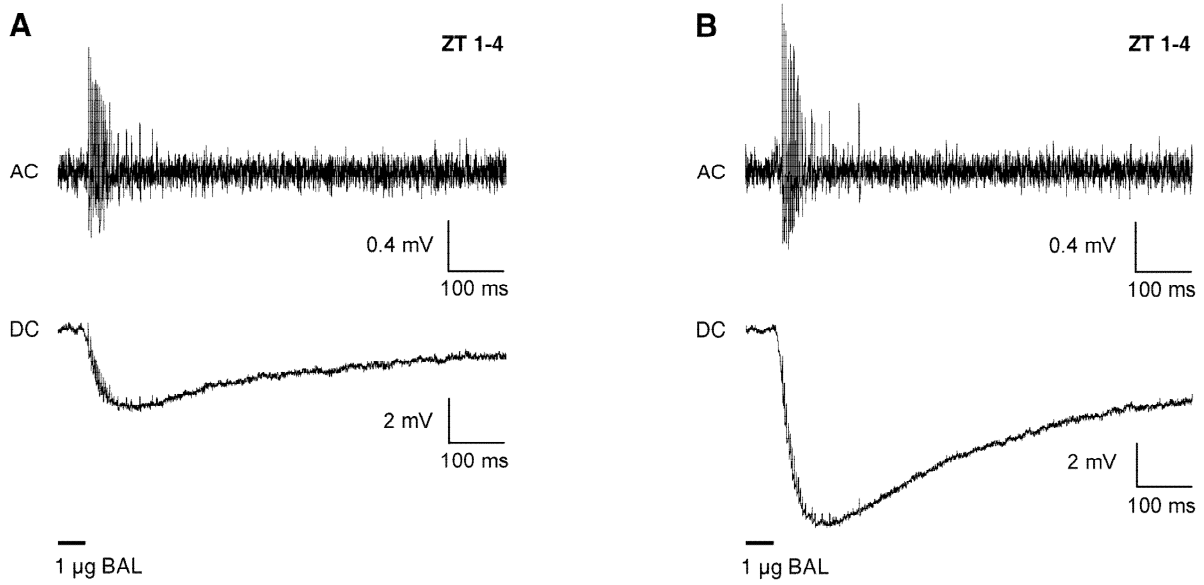

Fig. 1: Perfusion with 10 mmol^{-1} 8bcAMP increased the BAL-dependent SP amplitude, without affecting the initial AP frequency. Single responses to stimulation with 10 µg BAL at ZT 1-4 from the beginning (**A**) and end (**B**) of the recording duration of 180 min were compared. The observed increase in the AP amplitude was not 8bcAMP-dependent.

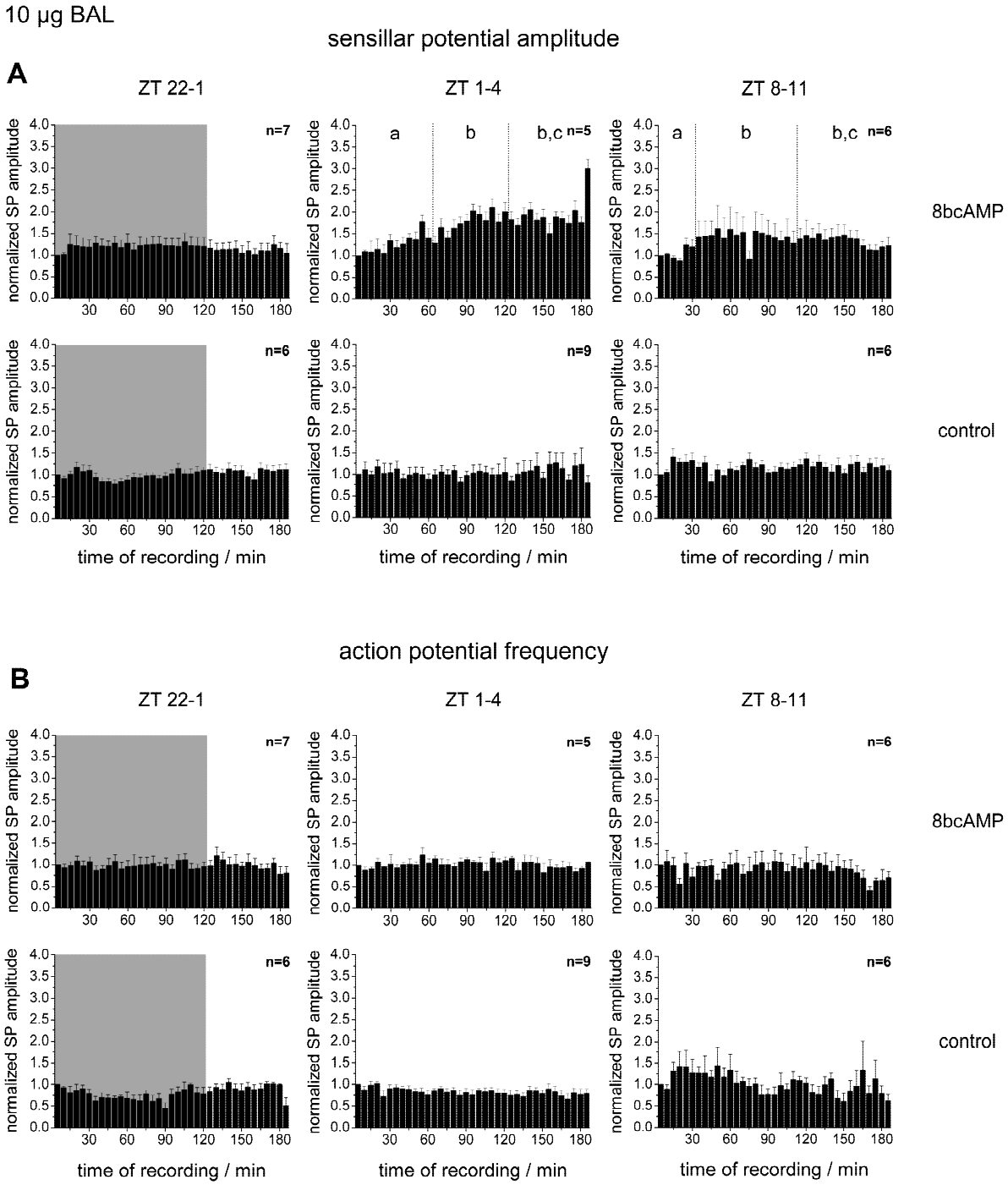

Fig. 2: 8bcAMP increased the SP amplitude in recordings with 10 µg BAL stimuli at ZT 1-4 and ZT 8-11. Analysis of the normalized and binned SP amplitude (**A**) and initial AP frequency (**B**) (computed over the first 5 ISI) for recordings with 10 µg BAL stimulation at ZT 22-1, ZT 1-4 and ZT 8-11. Values are mean + s.e.m. The perfusion with 10 $mmol^{-1}$ 8bcAMP increased the SP amplitude significantly at ZT 1-4 and ZT 8-11, when compared to the controls (Student´s *t*-test for independent samples, $P<0.001$ or Mann-Whitney-Test, $P<0.001$) and when comparing the intervals within each affected time course (ANOVA and Tukey HSD post-hoc test; $\alpha=0.001$, $P<0.001$ or Mann-Whitney-Test, $P<0.01$). The 8bcAMP-dependent increase at ZT 1-4 was 1.3-fold stronger than at ZT 8-11. The perfusion with 8bcAMP did not affect the initial AP frequency at any ZT. In the control recordings no significant changes in the time course of the SP amplitude and the AP frequency were observed for any ZT. *Different lower case letters* denote significant differences between tested groups of mean values.

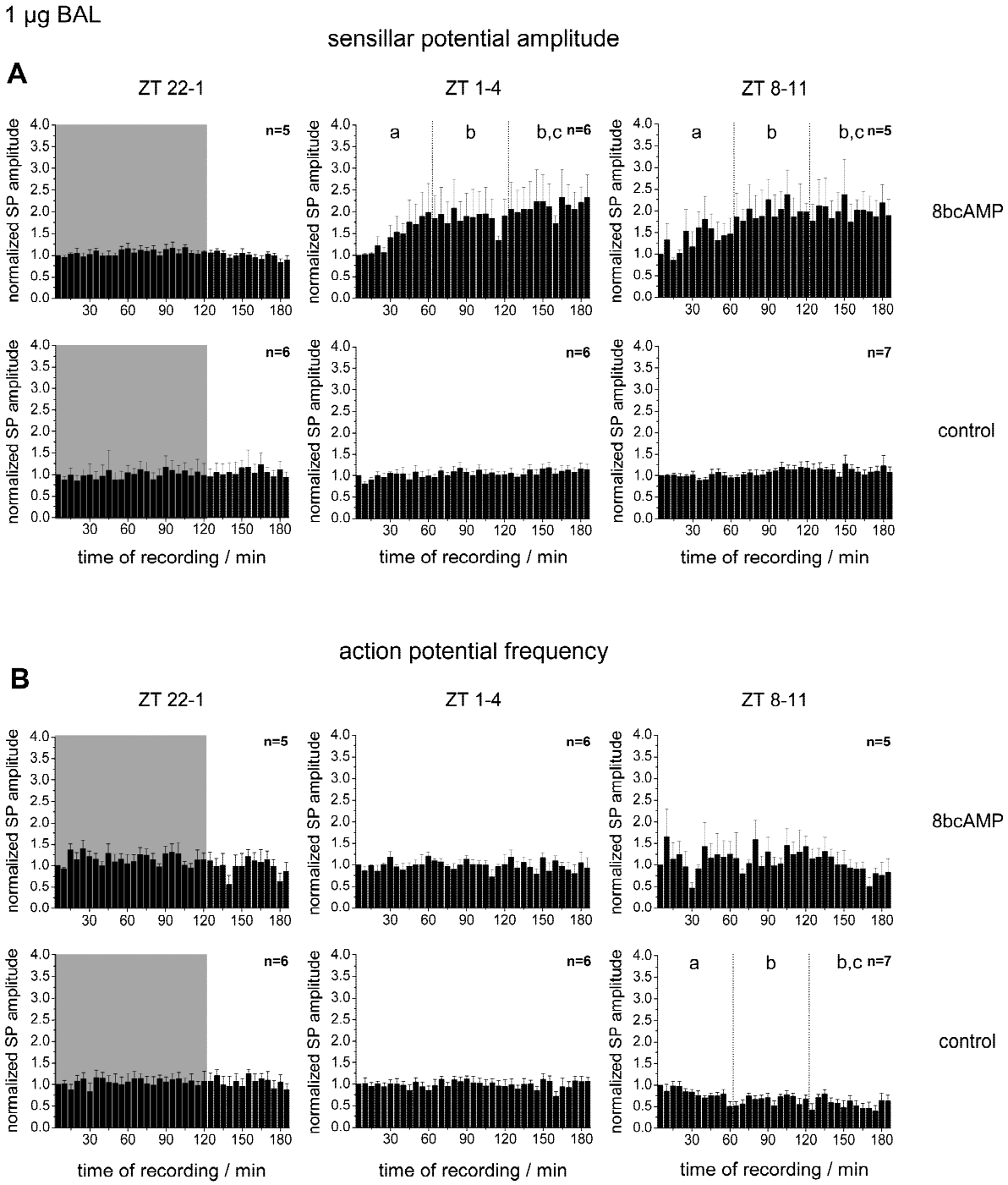

Fig. 3: In recordings with 1 µg BAL stimuli 8bcAMP increased the SP amplitude but only during the photophase. Comparison of the normalized and binned SP amplitude (**A**) and initial AP frequency (**B**) for recordings with 1 µg BAL stimulation at ZT 22-1, ZT 1-4 and ZT 8-11. Values are mean + s.e.m. The perfusion with 10 $mmol^{-1}$ 8bcAMP increased the SP amplitude with a similar time course and strength at ZT 1-4 and ZT 8-11. The increases were significant in comparison to the controls (Mann-Whitney-Test, $P<0.001$) and when comparing the intervals within each affected time course (Mann-Whitney-Test, $P<0.01$). In the control recordings at ZT 22-1, ZT 1-4 and ZT 8-11 no effects on the SP amplitude were observed. In contrast, the AP frequency in the control recordings decreased significantly at ZT 8-11, but not at ZT 22-1 or ZT 1-4 (ANOVA and Tukey HSD post-hoc test; $\alpha=0.01$, $P<0.01$). *Different lower case letters* denote significant differences between tested groups of mean values.

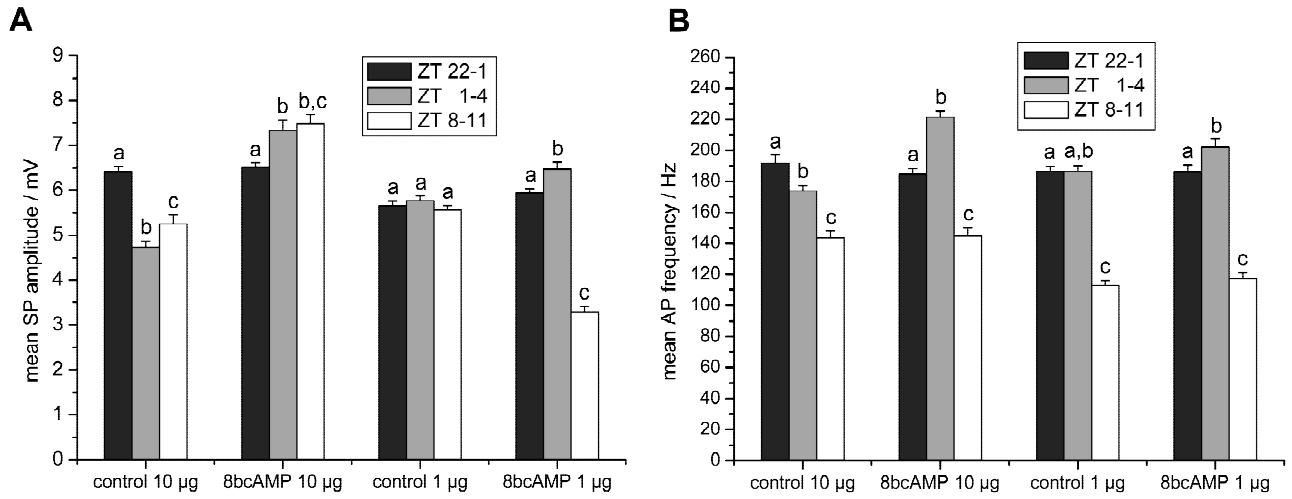

Fig. 4: Time- and pheromone-dose-dependent changes for control and 8bcAMP recordings were observed in the not normalized mean SP amplitude (**A**) and mean AP frequency (**B**) calculated over all responses for ZT 22-1, ZT 1-4 and ZT 8-11. Values are mean + s.e.m. The mean SP amplitude in control recordings with 10 µg BAL stimulation was decreased significantly at ZT 1-4 and ZT 8-11 when compared to ZT 22-1 (Mann-Whitney-Test, $P<0.001$). In controls employing 1 µg BAL stimulation no differences were found between the three ZTs. The application of 8bcAMP increased the mean SP amplitude in recordings with 10 µg BAL stimulation at ZT 1-4 and ZT 8-11 (Mann-Whitney-Test, $P<0.001$). Also in 8bcAMP recordings with 1 µg BAL stimulation a significant increase in the SP amplitude was found at ZT 1-4; at ZT 8-11 however a strong decrease in the mean SP amplitude was observed (Mann-Whitney-Test, $P<0.001$). As previously shown (Flecke and Stengl, 2009), in both controls with 10 and 1 µg BAL stimulation significant decreases in the mean AP frequency (**B**) were found at ZT 8-11 when compared to ZT 22-1 (Mann-Whitney-Test, $P<0.001$). Also in the recordings with 8bcAMP application significant decreases in the mean AP frequency were observed at ZT 8-11 for recordings employing 10 and 1 µg BAL stimuli (Mann-Whitney-Test, $P<0.001$). In contrast, 8bcAMP increased the mean AP frequency at ZT 1-4 for recordings with 10 and 1 µg BAL stimulation (Mann-Whitney-Test, $P<0.001$). *Different lower case letters* denote significant differences between tested groups of mean values.

In contrast, in controls with 1 µg BAL stimulation no significant differences were found between the three ZTs. The perfusion of 8bcAMP in recordings with 10 µg BAL stimulation increased the mean SP amplitude significantly at ZT 1-4 and ZT 8-11 (Fig. 4A) (Mann-Whitney-Test, $P<0.001$). In contrast, in 8bcAMP recordings employing 1 µg BAL the mean SP amplitude was only increased at ZT 1-4 but decreased at ZT 8-11 (Mann-Whitney-Test, $P<0.001$). As previously shown (Flecke and Stengl, 2009), in controls with 10 and 1 µg BAL stimulation significant decreases in the mean AP frequency occurred at ZT 8-11 (Fig. 4B) (Mann-Whitney-Test, $P<0.001$). Also in 8bcAMP recordings with 10 and 1 µg BAL stimulation decreases in the mean AP frequency were found at ZT 8-11 (Fig. 4B) (Mann-Whitney-Test, $P<0.001$), confirming the lack of 8bcAMP-dependent effects on the AP response observed for the normalized time courses. In addition, in recordings with 10 and 1 µg BAL stimulation 8bcAMP significantly increased the mean AP frequency at ZT 1-4 (Mann-Whitney-Test, $P<0.001$). Time-dependent changes in the mean SP amplitude were not always followed by consistent changes in the mean AP frequency, especially at ZT 8-11. Although in 8bcAMP recordings with 10 µg BAL stimulation an increase in the mean SP amplitude was observed at ZT 8-11, the shift to lower mean AP frequencies at ZT 8-11 was not antagonized by this increase. Also in control recordings with 1 µg BAL stimulation the mean AP frequency was significantly decreased at ZT 8-11 (Fig. 4B) without a consistent decrease in the mean SP amplitude at ZT 8-11 (Fig. 4A). The absolute values of the mean SP amplitude and the mean AP frequency measured at ZT 22-1 were in the same range for all 8bcAMP and control recordings of equal BAL dose (Tabs. 1, 2), confirming similar conditions during the experiments.

Table 1 Mean SP amplitude (+/- s.e.m.) in BAL responses for three ZTs

	control 10 µg BAL	**8bcAMP 10 µg BAL**	**control 1 µg BAL**	**8bcAMP 1 µg BAL**
ZT 22-1	6.39 +/- 0.13 (n=212)	6.52 +/- 0.11 (n=246)	5.64 +/- 0.11 (n=187)	5.92 +/- 0.11 (n=187)
ZT 1-4	4.72 +/- 0.12 (n=201)	7.34 +/- 0.21 (n=132)	5.76 +/- 0.11 (n=179)	6.47 +/- 0.14 (n=206)
ZT 8-11	5.24 +/- 0.2 (n=196)	7.49 +/- 0.19 (n=207)	5.57 +/- 0.1 (n=217)	3.28 +/- 0.12 (n=165)

Table 2 Mean AP frequency (+/- s.e.m.) in BAL responses for three ZTs

	control 10 µg BAL	8bcAMP 10 µg BAL	control 1 µg BAL	8bcAMP 1 µg BAL
ZT 22-1	191.65 +/- 5.54 (n=188)	184.35 +/- 3.83 (n=243)	186.38 +/- 3.45 (n=225)	186.1 +/- 4.47 (n=181)
ZT 1-4	173.57 +/- 3.87 (n=283)	221.34 +/- 3.91 (n=144)	186.61 +/- 3.51 (n=216)	202.1 +/- 5.24 (n=200)
ZT 8-11	143.37 +/- 4.68 (n=191)	144.85 +/- 5.18 (n=204)	112.87 +/- 3.29 (n=251)	117.23 +/- 4.12 (n=164)

Effects on the AP distribution in BAL responses

Time-dependent differences were also found in the distribution of APs in BAL responses. The distribution of APs during the first 1000 ms of the responses were analyzed by creating PSTHs for each ZT for responses at the beginning (0-20 min), middle (80-100 min) and end (160-180 min) of the recording duration. The PSTHs were analyzed because they include information about the temporal resolution of pheromone pulse detection, since only ORNs with phasic responses can follow odour pulses with high frequencies. First it was examined whether the previously observed ZT-dependent shifts in the response patterns and in the number of APs in the first 100 ms of the responses were affected by 8bcAMP. Then it was examined, whether the shift in the response pattern with/without 8bcAMP depends on the pheromone concentration employed. As previously shown, in control recordings with 10 µg BAL stimulation the responses at ZT 8-11 were shifted from a phasic to a tonic response pattern (Fig. 6A) and the number of APs in the first 100 ms decreased slightly at ZT 1-4 (Fig. 5B) and strongly at ZT 8-11 (Fig. 6 AB) (ANOVA and Tukey HSD post-hoc test; α=0.01, P<0.01) (Flecke et al., 2006). Also in recordings with 1 µg BAL stimulation similar shifts in the response distribution and significant decreases in the number of APs during the first 100 ms of the responses were found at ZT 1-4 and ZT 8-11 (Figs. 5AB, 6AB) (ANOVA and Tukey HSD post-hoc test; α=0.01, P<0.01 or Mann-Whitney-Test, P<0.01). In contrast, under the influence of 8bcAMP at ZT 1-4 no significant shift to more tonic responses (Fig. 5 A) and no decrease in the number of APs in the first 100 ms were found with 10 and 1 µg BAL stimulation (Fig. 5B) (Mann-Whitney-Test, P>0.01). However, the PSTHs from the end of the recording duration (Fig. 5A) and the time courses of the number of APs over the first 100 ms of 8bcAMP and control recordings with 1 µg BAL stimulation (Fig. 5B) were not significantly different from each other (Mann-Whitney-Test, P>0.05). Only in recordings with 10 µg BAL stimulation significant differences were found between 8bcAMP and control recordings (Mann-Whitney-Test, P<0.001). These differences resulted from the relatively weak responses at the beginning of the 8bcAMP recordings and from the fewer repetitions in 8bcAMP recordings. In recordings at ZT 8-11, the application of 8bcAMP did not have a significant effect on the AP distribution (Fig. 6 AB). As in the associated control recordings also with 10 $mmol^{-1}$ 8bcAMP and 10 µg BAL stimuli the AP distribution was shifted from phasic to tonic response patterns (Fig. 6A) and the number of APs in the first 100 ms of the responses decreased significantly (Fig. 6B) (ANOVA and Tukey HSD post-hoc test; α=0.01, P<0.01). The PSTHs of 8bcAMP and control recordings with 10 µg BAL stimulation from the end of the recordings did not differ significantly from each other (Fig. 6A) (Mann-Whitney-Test, P>0.05). In addition, no significant differences were found in the comparison of the time courses of the mean number of APs in the first 100 ms of 8bcAMP and control recordings with 10 µg BAL stimuli (Fig. 6B) (Student´s t-test for independent samples, P>0.05). In contrast, in 8bcAMP recordings with 1 µg BAL stimulation at ZT 8-11 no clear shift from phasic to tonic responses was measured (Fig. 6A). Although, the comparison of the PSTHs of 8bcAMP and control recordings from the end of the recording duration revealed no significant differences (Mann-Whitney-Test, P>0.05), no decrease in the number of APs in the first 100 ms was found for 8bcAMP recordings. This was due to the relatively weak responses at the beginning of the recording duration (Fig. 6A). Nonetheless, consistently all PSTHs from the end of the recording duration at ZT 8-11 were found to show less phasic AP distributions.

Effects on the spontaneous AP frequency

In addition to the effects on the responses to stimulation with BAL, 8bcAMP also affected the generation of spontaneous APs recorded between the stimulations. In recordings at ZT 8-11 and with 1 µg BAL stimulation the perfusion with 8bcAMP was followed by a fast increase in the normalized spontaneous AP frequency (Fig. 7) (Mann-Whitney-Test, P<0.01). The 8bcAMP-dependent increase in the normalized spontaneous AP frequency was caused by equal increases in the number of bursts per bin and the number of spikes per bin (Fig. 7) (ANOVA and Tukey HSD post-hoc test; α=0.01, P<0.01), without affecting the percentage of APs occurring in bursts or the average number of APs per burst. All 8bcAMP-dependent effects were significant in the comparison to the controls (Mann-Whitney-Test, P<0.001 or Student's t-test for independent samples, P<0.001). In accordance with recently published results (Flecke and Stengl, 2009) (Fig. 8) the normalized spontaneous AP frequency was slightly but significantly decreased in the control recordings employing 1 µg BAL stimuli (Fig. 7) (Mann-Whitney-Test, P<0.01). In addition, the number of bursts and the number of spikes were decreased significantly in the controls (ANOVA and Tukey HSD post-hoc test; α=0.01, P<0.01). Also in the controls with 10 µg BAL stimulation the normalized spontaneous AP frequency, the number of bursts per bin and the number

of spikes per bin decreased significantly (Fig. 8) (Mann-Whitney-Test, $P<0.001$). In recordings with 8bcAMP perfusion employing 10 µg BAL no significant increase was found for the normalized spontaneous AP frequency. However, the 8bcAMP-dependent, stable time courses of the normalized spontaneous AP frequency and the number of spikes per bin differed significantly from the parameters of the associated controls (Mann-Whitney-Test, $P<0.01$).

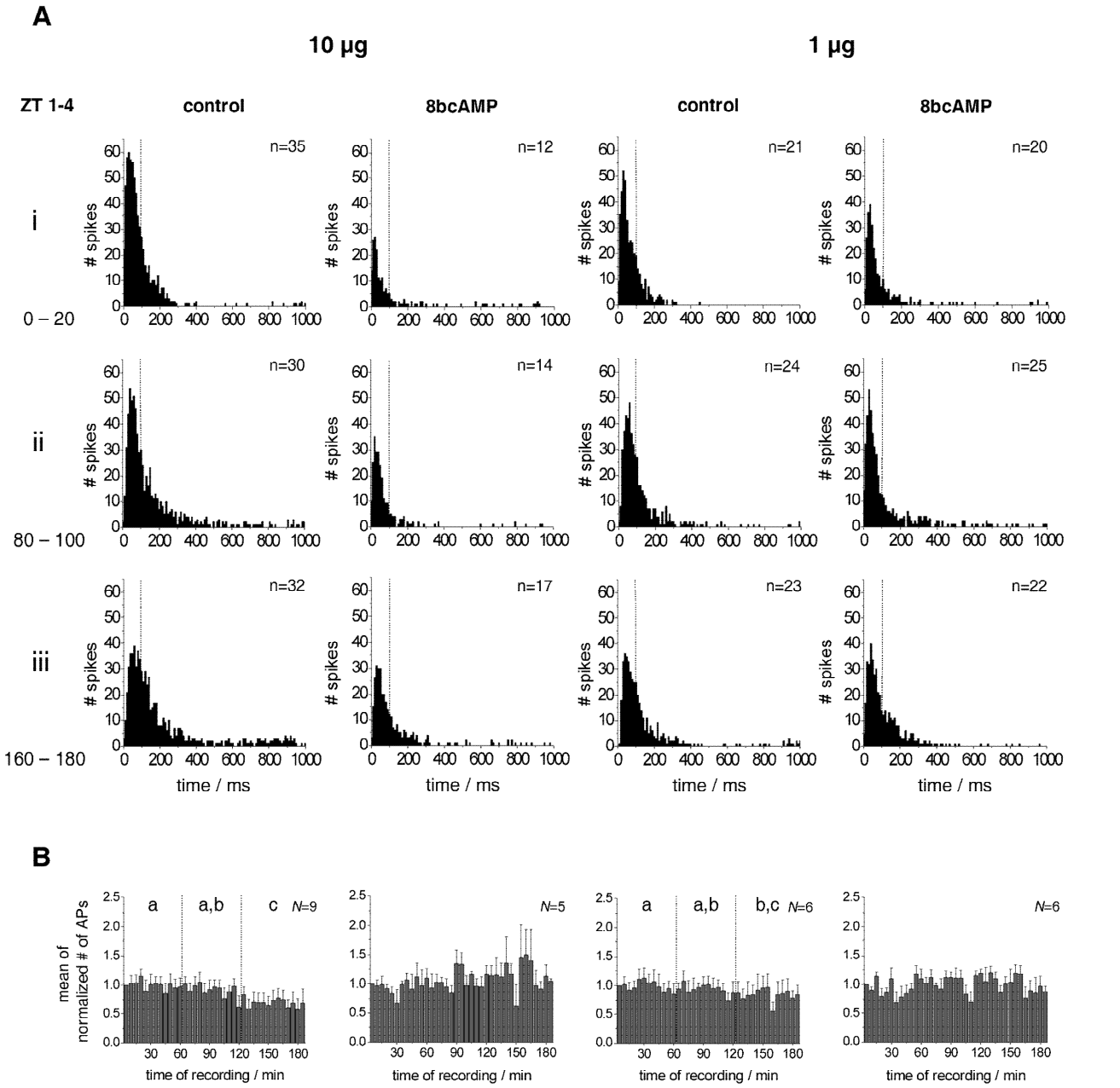

Fig. 5: Perfusion with 8bcAMP did not the affect the kinetics of BAL responses at ZT 1-4. PSTHs (**A**) from the beginning (0-20 min), middle (80-100 min) and end (160-180 min) for recordings with 10 (**left**) or 1 µg (**right**) BAL stimulation at ZT 1-4 and the mean (+ s.e.m.) of the normalized number of APs (**B**) elicited in the first 100 ms of the responses for 180 min of the recording duration at ZT 1-4. In the control recordings with 10 and 1 µg BAL stimulation a slight shift from phasic to tonic responses and also a decrease in the number of APs in the first 100 ms was found (ANOVA and Tukey HSD post-hoc test; $\alpha=0.01$, $P<0.01$). In recordings with application of 8bcAMP at both doses no significant variations over the recording duration were observed in the AP distribution and in the number of APs in the first 100 ms of the responses. The PSTHs from the end of the recordings of both 8bcAMP- and control recordings with 1 µg BAL stimulation and the time courses of the number of APs in the first 100 ms of both data sets were not significantly different from each other (Mann-Whitney-Test, $P>0.05$). Only between 8bcAMP- and control recordings with 10 µg BAL stimulation significant differences were found (Mann-Whitney-Test, $P<0.001$), that are the result of the weak BAL responses and the lower number of repetitions in the 8bcAMP recordings. *Different lower case letters* denote significant differences between tested groups of mean values.

To determine whether 8bcAMP affected the mean spontaneous AP frequency calculated for the whole data set at each ZT, different ZTs of control and 8bcAMP recordings were compared (Fig. 9). In the control recordings with 10 and 1 µg BAL stimuli significant and gradual decreases in the mean spontaneous AP frequency occurred at ZT 1-4 and ZT 8-11 (Fig. 9A) (Mann-Whitney-Test, $P<0.001$). Perfusion with 8bcAMP antagonized this shift at ZT 1-4 and ZT 8-11, since no significant differences were found between the three ZTs for 8bcAMP recordings with 10 and 1 µg BAL stimulation. In addition, OA counteracted the decrease to a lower mean spontaneous AP frequency at ZT 1-4 and significantly increased the mean spontaneous AP frequency at ZT 8-11 (Fig. 9A) (Mann-Whitney-Test, $P<0.001$). To analyze the origin of the effects on the mean spontaneous AP frequency the mean number of bursts per bin (Fig. 9B) and mean number of spikes per bin (Fig. 9C) were evaluated for the three ZTs. In accordance to the shift of the mean spontaneous AP frequency, the mean number of bursts (Fig. 9B) and the mean number of spikes (Fig. 9C) were significantly decreased in control recordings with 1 µg BAL stimuli at ZT 1-4 and ZT 8-11 (Mann-Whitney-Test, $P<0.001$). In control recordings employing 10 µg BAL stimulation the decreases in the mean number of bursts and spikes were only significant at ZT 8-11. The application of 8bcAMP antagonized the shift to lower mean number of burst (Fig. 9B) and spikes (Fig. 9C) in recordings with both stimulus doses, since no significant differences were found between the three ZTs. Furthermore, OA counteracted the shift to lower mean number of bursts (Fig. 9B) and spikes (Fig. 9C) at ZT 1-4 and significantly increased both parameters at ZT 8-11. The OA-dependent increase in the mean number of bursts was 1.4-fold stronger than the increase in the mean number of spikes. No significant differences between the ZTs were found for the mean average APs per burst and the mean percentage of APs in bursts (data not shown).

Discussion

To investigate whether 8bcAMP mimics OA-dependent disadaptation of ORNs of *M. sexta* during the photophase and to examine the role of cAMP in pheromone transduction long-term tip recordings were performed at three ZTs (ZT 22-1, ZT 1-4 and ZT 8-11) and at two different BAL doses. Perfusion with 8bcAMP increased the normalized SP amplitude in responses to stimulation with 10 and 1 µg BAL in recordings at ZT 1-4 and ZT 8-11, but not at ZT 22-1. In addition, 8bcAMP increased the mean SP amplitude calculated over the absolute values at ZT 1-4 and ZT 8-11 for recordings with 10 µg BAL stimulation and at ZT 1-4 for recordings employing 1 µg BAL. In contrast, 8bcAMP did not affect the normalized initial AP frequency, the distribution of APs in responses to stimulation with BAL and did not antagonize the shift to lower mean AP frequencies at ZT 8-11. Furthermore, 8bcAMP increased the normalized spontaneous AP frequency in recordings with 1 µg BAL stimulation at ZT 8-11 by increasing both the number of spikes and the number of bursts. 8bcAMP also affected the mean spontaneous AP activity by antagonizing shifts to lower mean spontaneous AP frequencies, to lower number of bursts and to lower number of spikes at ZT 1-4 and ZT 8-11. In addition, perfusion with OA antagonized shifts to lower mean spontaneous AP frequencies at ZT 1-4 and strongly increased the mean spontaneous AP frequency at ZT 8-11 by predominantly increasing the mean number of bursts.

8bcAMP affects pheromone transduction but does not mimic all OA effects

To our knowledge, we showed for the first time that cAMP is involved in the modulation of the SP of single pheromone-sensitive trichoid sensilla of a moth species. Our results are in agreement with a previous study which showed that perfusion with cAMP, with its analouges or phosphodiesterase inhibitors increased pheromone-dependent EAG amplitudes in antennae of *A. polyphemus* (Villet, 1978). Also in *D. melanogaster* evidence was found for cAMP-dependent modulation of insect odour transduction. It was shown that in *dunce*-mutants, which overexpressed a cAMP-phosphodiesterase, rise-time kinetics in EAG recordings (Martín et al., 2001) were slowed down and the sensitivity to acetone and ethanol in behavioural y-maze experiments was decreased (Gomez-Diaz et al., 2004). Recently, we showed that application of OA with the same experimental protocol increased the SP amplitude even stronger than 8bcAMP but with a similar time course (Flecke and Stengl, 2009). In contrast to 8bcAMP, OA also increased the normalized initial AP frequency, antagonized an endogenous shift to lower mean AP frequencies at ZT 8-11 and antagonized an endogenous shift to more tonic pheromone responses at ZT 8-11 (Flecke and Stengl, 2009). Thus, the application of 8bcAMP only partly mimics the OA-dependent effects. The majority of insect OA receptors were shown to be positively coupled to adenylyl cyclases (Evans and Maqueira, 2005; Farooqui, 2007). In addition an OA-receptor was cloned from *M. sexta* and localized in the antennae (Dacks et al., 2006). The receptor was not pharmacologically characterised yet, but has a high sequence-similarity to other insect OA α-adrenergic-like receptors, that were shown to increase both intracellular cAMP- and Ca^{2+}-levels. Therefore, and due to the similarity of 8bcAMP- and OA-dependent effects on the SP it is likely that the involved OA receptor also activates an adenylyl cyclase.

Next to the modulation of odour transduction cAMP might also be directly involved in the odour transduction of some general odours in the fruit fly as was suggested by Wicher et al. (2008). In *D. melanogaster* the stimulation of the heterologously expressed olfactory receptor OR22a activated an adenylyl cyclase. Increased levels of cAMP then activated the ubiquitous OR83b inducing an additional inward current. Thus, the cyclic

nucleotide sensitive binding site on OR83b is a potential target for 8bcAMP- and OA-dependent modulation of the primary olfactory transduction process not only of general odours but also of pheromones. Although the cAMP-dependent current observed by Wicher et al (2008) showed a very late activation after the stimulation (peak at 60 s) it is possible that this current also contributes to the generation of the SP. More likely is however, that cAMP is involved in

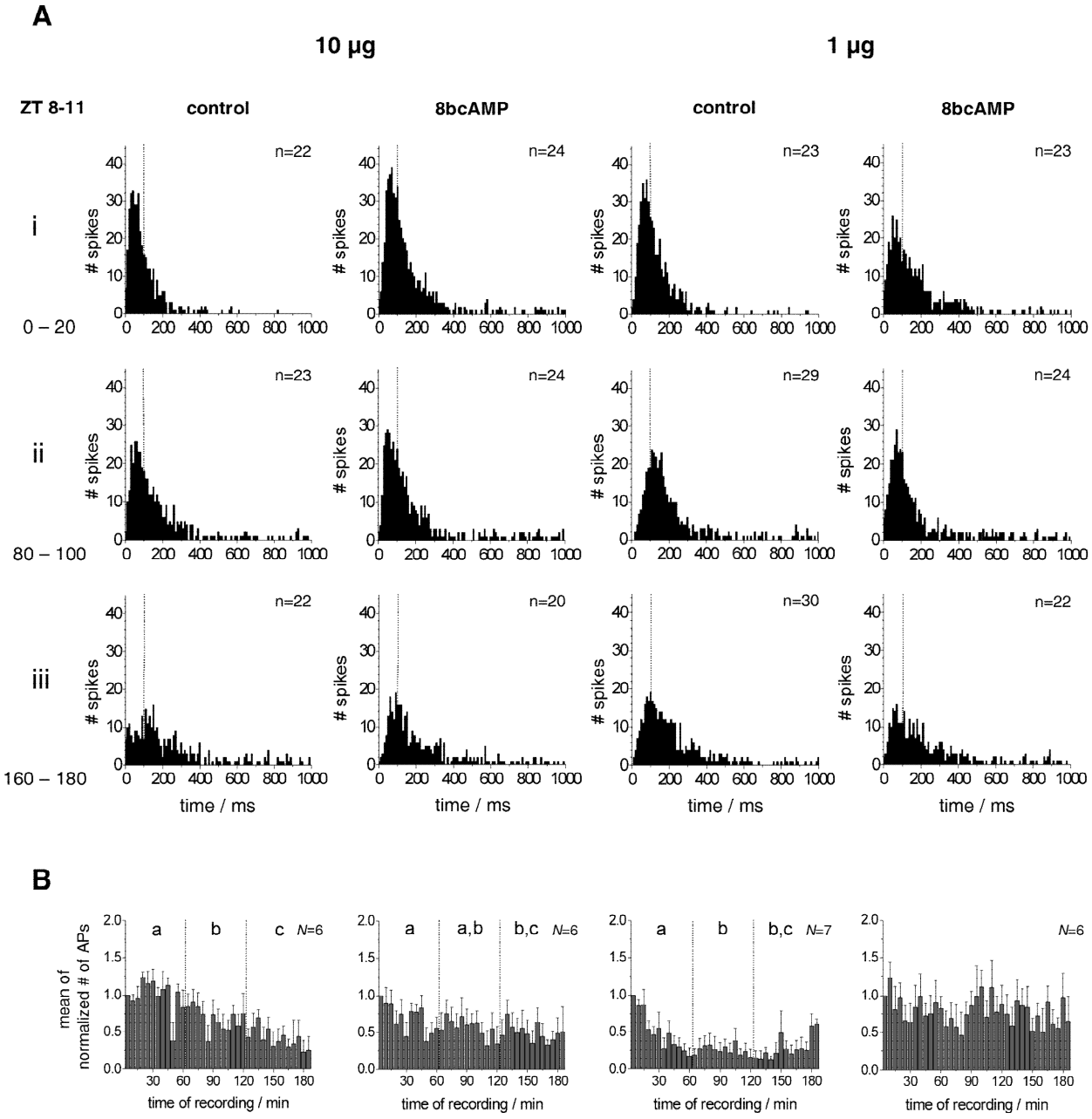

Fig. 6: Perfusion with 8bcAMP did not affect the endogenous shift to tonic BAL responses at ZT 8-11. PSTHs (**A**) from the beginning (0-20 min), middle (80-100 min) and end (160-180 min) of the recording duration for recordings with 10 (**left**) or 1 µg (**right**) BAL stimulation at ZT 8-11. In the controls AP responses to both BAL doses showed a strong shift from phasic to tonic response patterns. Also the mean (+ s.e.m.) of the number of APs which occurred in the first 100 ms of the responses (**B**) were significantly decreased (ANOVA and Tukey HSD post-hoc test; α=0.01, P<0.01 or Mann-Whitney-Test, P<0.01). During 8bcAMP application a comparable and significant shift to tonic responses and a decrease in the number of APs in the first 100 ms was only found in recordings with 10 µg BAL stimuli (**left**) (ANOVA and Tukey HSD post-hoc test; α=0.01, P<0.01). Although in recordings with 1 µg BAL stimulation the PSTHs of 8bcAMP and control recordings from the end of the recordings were not significantly different (Mann-Whitney-Test, P>0.05), due to the weak responses at the beginning of the 8bcAMP recordings no decrease in the number of APs in the first 100 ms was observed. *Different lower case letters* denote significant differences between tested groups of mean values.

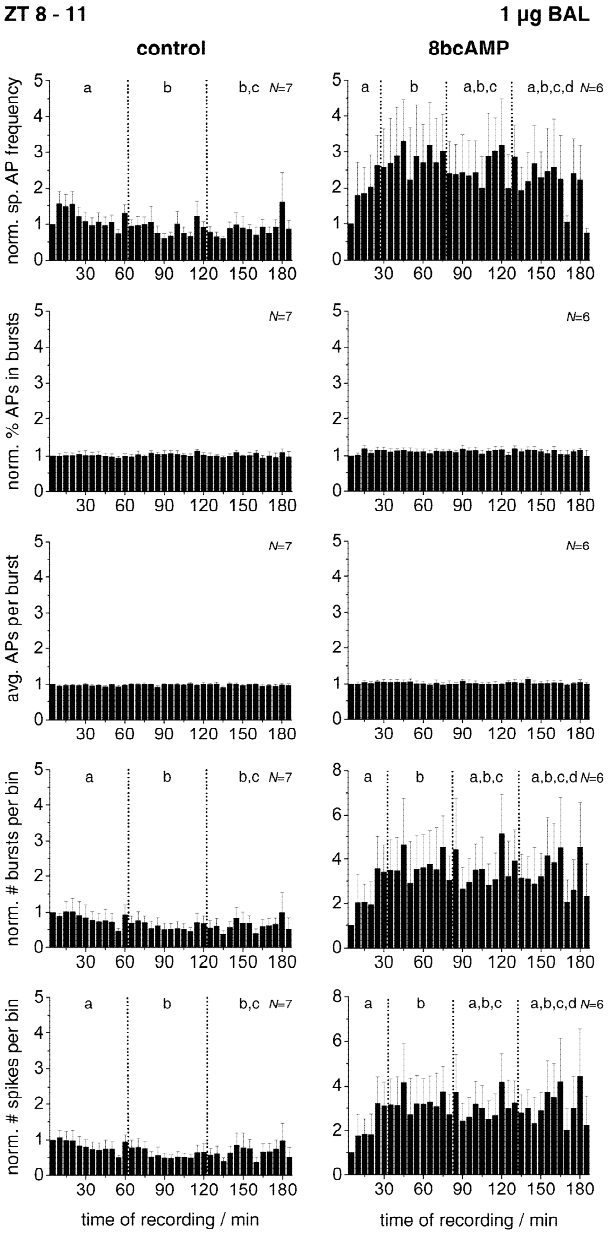

Fig. 7: In recordings at ZT 8-11 employing 1 µg BAL stimuli perfusion with 8bcAMP significantly increased the normalized spontaneous AP frequency, the number of bursts per bin and the number of spikes per bin with very similar time courses (Mann-Whitney-Test, $P<0.01$ or ANOVA and Tukey HSD post-hoc test; $\alpha=0.01$, $P<0.01$). In contrast, in the associated controls slight but significant decreases were found for the spontaneous AP frequency, the number of bursts per bin and the number of spikes per bin (Mann-Whitney-Test, $P<0.01$ or ANOVA and Tukey HSD post-hoc test; $\alpha=0.01$, $P<0.01$). Values are mean + s.e.m. *Different lower case letters* denote significant differences between tested groups of mean values.

the slow modulation of the primary transduction process. This assumption is further supported by another study, which showed that inhibitors of adenylyl cyclases and of G-proteins had no influence on the initial maximal Ca^{2+}-increase after odour stimulation but significantly affected the rectification to baseline levels at a point in time after stimulation when the cAMP-dependent current was shown to occur (Smart et al., 2008; Wicher et al., 2008). In addition, it is still unknown whether this cAMP-dependent current also is involved in odour transduction *in vivo* and whether the OR83b analogue MsextaOR2 (Patch et al., 2009) also has a cAMP-sensitive binding site.

Next to a direct cAMP-dependent modulation of ion channels cAMP might also act indirectly via a protein kinase A, phosphorylating cation channels in the outer dendritic segment. This could provide more potential targets, finally resulting in an increased receptor current. So far, an apparently directly cAMP-dependent nonspecific cation channel and a cAMP-dependent Ca^{2+}-channel were identified in *M. sexta* (Krannich and Stengl, 2008), next to cation and potassium channels (Dolzer, 2002). Preliminary experiments with the application of 1 mmol l^{-1} serotonin showed no effects, thus the upregulation of cAMP via a pathway involving serotonin receptors is unlikely (unpublished results).

An additional explanation for the increases in the SP amplitude could be an effect of 8bcAMP on V-ATPases located in the accessory cells of trichoid sensilla of moth species (Klein and Zimmermann, 1991; Klein, 1992; Beyenbach and Wieczorek, 2006). For *M. sexta* it was shown that in the epithelium of the midgut the H^+ gradient created by the activity of V-ATPases powers a K^+/nH^+ antiporter which builds up the high K^+ concentration in the extracellular space necessary for the generation of the TEP (Wieczorek et al., 1991; Wieczorek, 1992; Wieczorek et al., 2003). Also the TEP in trichoid sensilla of moth is supposed to be generated by an active K^+ transport through the apical membranes of the accessory cells into the sensillum lymph cavity (Thurm, 1972; Thurm, 1974). The high K^+ concentration in the sensillum lymph causes a potential difference between the sensillum lymph and the hemolymph. This potential difference is assumed to add to the membrane potential of the outer dendrites. In addition, it was shown, that the application of cAMP and forskolin on salivary glands of the blowfly significantly increased V-ATPase activity (Dames et al., 2006) and altered the TEP (Prince and Berridge, 1972; Berridge and Prince, 1972). Thus, it is possible that cAMP modulated the V-ATPase activity in accessory cells, finally increasing the driving force for the SP. If V-ATPases in accessory cells of *M. sexta* in fact are cAMP-sensitive and if changes in the TEP could indirectly regulate the amplitude of the SP remains to be studied.

SP amplitude increase without strong effects on the AP frequency

8bcAMP strongly affected the normalized SP amplitude at ZT 8-11 and ZT 1-4 but had only minor effects on the generation of BAL-dependent APs. In addition, 8bcAMP did not antagonize the endogenous shift to lower mean AP

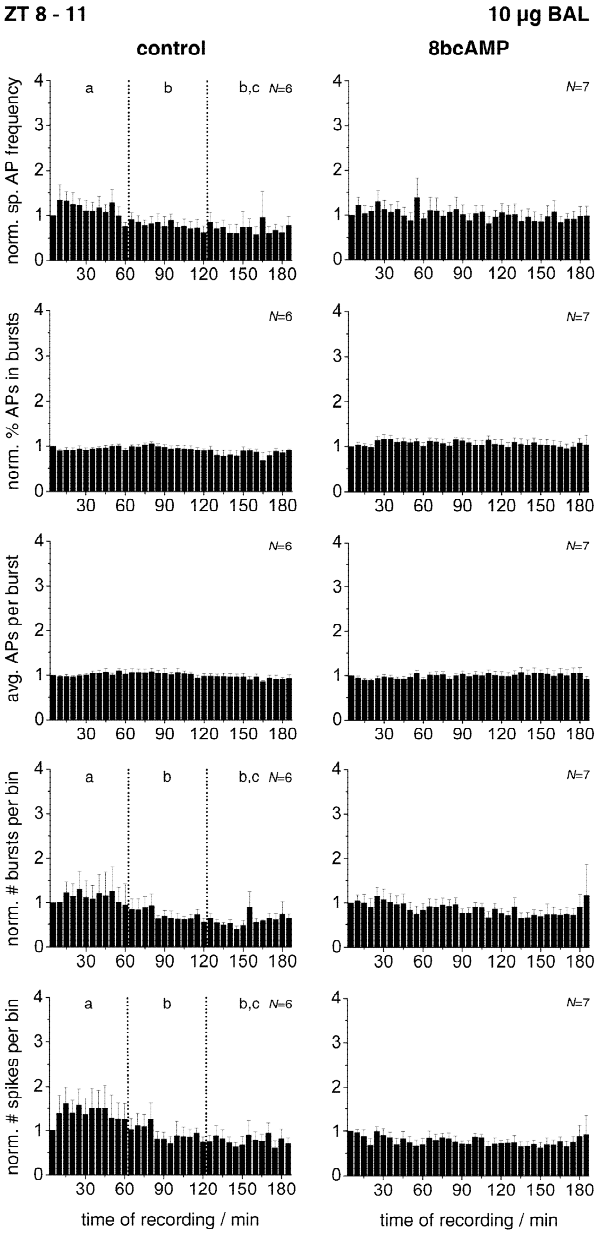

Fig. 8: In recordings at ZT 8-11 employing 10 µg BAL stimuli perfusion with 8bcAMP antagonized a decrease of the normalized spontaneous AP frequency. In the controls the normalized spontaneous AP frequency, the number of bursts per bin and the number of spikes per bin decreased slightly but significantly (Mann-Whitney-Test, $P<0.001$). The stable 8bcAMP-dependent time courses of the normalized spontaneous AP frequency and the number of spikes per bin were shown to be significantly different from the controls (Mann-Whitney-Test, $P<0.01$). Values are mean + s.e.m. *Different lower case letters* denote significant differences between tested groups of mean values.

frequencies at ZT 8-11. In contrast to OA, 8bcAMP had only negligible effects on the pheromone pulse resolution of the ORNs and did not antagonize an endogenous shift to more tonic responses at ZT 8-11. Only at ZT 1-4 in recordings with 1 and 10 µg BAL stimuli increases in the mean SP amplitude were followed by increases in the mean AP frequency. Remarkably, the increase in the normalized SP amplitude, which is apparently due to a further depolarization of the dendrite, was not transformed into an equal/comparable elevation of the normalized AP frequency. Thus, either the 8bcAMP-dependent augmentation of the receptor current did not reach the AP triggering zone or an additional adaptation mechanism prevented the increase in the normalized AP frequency. Previous investigations of adaptation processes in trichoid sensilla of *M. sexta* indicated the existence of additional adaptation mechanisms at the level of the AP response (Dolzer et al., 2003). This is further supported by experiments in which the application of 8bcGMP only affected the AP response, but not the SP (Flecke et al., 2006). Furthermore, 8bcAMP did not increase the mean AP frequencies at ZT 8-11, although with 10 µg BAL an increase in the mean SP amplitude was found at this ZT. It remains to be examined whether the SP amplitude is additionally attenuated during the propagation towards the AP generator e.g. via an 8bcAMP-dependent activation of a hyperpolarization-activated cyclic nucleotide-gated (HCN, I_h) cation channel or other cyclic nucleotide sensitive channels which could decrease the input resistance locally as was shown for ORNs in the vomeronasal organ (Dibattista et al., 2008). In contrast to 8bcAMP, OA and tyramine increased both the SP amplitude and the AP frequency in tip recordings of trichoid sensilla (Flecke and Stengl, 2009). Thus, probably OA and tyramine activate more than one potential signalling pathway, affecting both the generation of the SP and its conversion into APs.

Several insect OA-receptors were found to increase both the level of cAMP and Ca^{2+} (Han et al., 1998; Bischof and Enan, 2004; Balfanz et al., 2005; Ohtani et al., 2006; Grohmann et al., 2003). In most cases it was shown that these Ca^{2+}-increases were independent of extracellular Ca^{2+}-concentrations, suggesting an inositol-1,4,5-triphosphate (IP_3) dependent release of Ca^{2+} from intracellular stores. Thus, the second potentially OA-dependent pathway could involve the activation of a phospholipase C, increasing the level of IP_3 and Ca^{2+}, which could be followed by a protein kinase C dependent phosphorylation of ion channels involved in AP generation. The 8bcAMP-dependent effects were rather slow, evolving during the first hour of the recordings. On this time scale a slow modulation due to a Ca^{2+}-increase appears to be possible. However, it is unlikely that such a process is involved in fast concentration-dependent in- or decreases in the AP frequency due to changes in the SP amplitude.

Effects on the spontaneous AP frequency

Perfusion with 8bcAMP increased the normalized spontaneous AP frequency in recordings with 1 µg BAL stimulation at ZT 8-11, but not in recordings with 10 µg BAL stimuli, which is most likely due to the weak 8bcAMP-effect on the normalized values in these

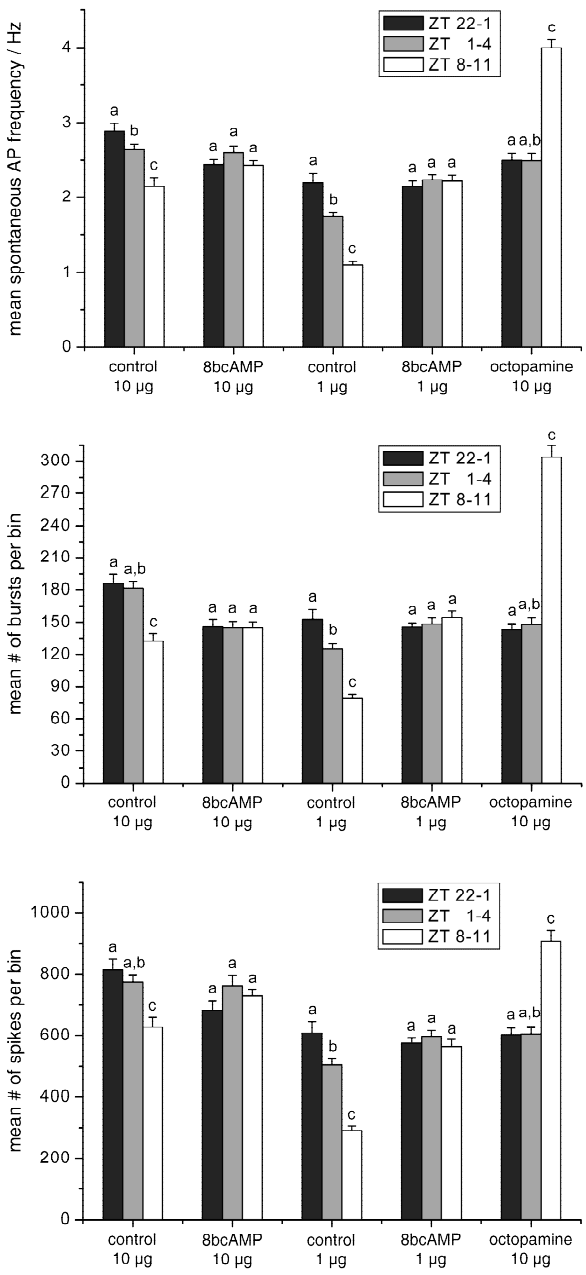

Fig. 9: Both 8bcAMP and OA antagonized endogenous shifts to a lower spontaneous AP activity during the photophase. The mean spontaneous AP frequency calculated over the absolute values for ZT 22-1, ZT 1-4 and ZT 8-11. All values are mean + s.e.m. Both in control recordings with 10 and 1 µg BAL stimulation the mean spontaneous AP frequency decreased gradually at ZT 1-4 and ZT 8-11 (Mann-Whitney-Test, $P<0.001$). In contrast, application of 8bcAMP in recordings with both BAL doses antagonized this shift at ZT 1-4 and ZT 8-11. The application of 1 mmol l^{-1} OA antagonized the decrease at ZT 1-4 and increased the mean spontaneous AP frequency significantly at ZT 8-11 (Mann-Whitney-Test, $P<0.001$). Significant decreases in the mean number of bursts per bin and the mean number of spikes per bin were found at ZT 8-11 for controls with 10 µg BAL stimulation and at ZT 1-4 and ZT 8-11 for controls employing 1 µg BAL (Mann-Whitney-Test, $P<0.001$). 8bcAMP antagonized the shift to lower mean number of bursts and mean number of spikes per bin. In addition, OA increased the mean number of bursts and spikes per bin significantly at ZT 8-11 (Mann-Whitney-Test, $P<0.001$). The OA-dependent increase in the mean number of bursts per bin was 1.4-fold stronger than the increase in the mean number of spikes per bin. *Different lower case letters* denote significant differences between tested groups of mean values.

recordings. In contrast to recently published OA-dependent effects on the spontaneous AP activity (Flecke and Stengl, 2009), 8bcAMP increased both the number of bursts and the number of spikes and did not affect the ratio of APs occurring as single spikes or in bursts nor did 8bcAMP increase the average number of APs per burst. In addition, 8bcAMP only antagonized the shift to lower mean spontaneous AP frequencies at ZT 1-4 and ZT 8-11, while OA strongly increased the mean spontaneous AP frequencies at ZT 8-11. OA appears to predominantly affect the burst behaviour of the ORNs, whereas 8bcAMP affects the generation of APs occurring as single spikes or in bursts to the same extent. If the hypothesis is correct that OA activates an adenylyl cyclase, additional targets might be activated by OA, which would lead to a further enhancement of the effects on the burst behaviour, as the 1.4-fold stronger OA-dependent effect on the mean number of bursts at ZT 8-11 suggests. Possibly, an OA-dependent additional increase in the intracellular Ca^{2+}-levels could affect endogenous oscillations in the Ca^{2+}-concentrations, which might affect bursts.

The 8bcAMP-dependent increase in the spontaneous AP frequency alone can be explained by the activation of a HCN cation channel. I_h currents were shown to be involved in the generation of spontaneous pacemaker activity and in the control of the resting potential (Robinson and Siegelbaum, 2003). Recently Dibattista et al. (2008) showed that blocking of I_h channels or blocking of adenylyl cyclases in ORNs of the vomeronasal organ decreased the resting potential and increased the current threshold to elicit APs. In addition, a decrease of the spontaneous AP activity by the blocking of an I_h current was observed in ORNs of *Panulirus argus* (Gisselmann et al., 2005). In contrast, the activation of adenylyl cyclases by forskolin increased the resting potential in ORNs of the vomeronasal organ by 9 mV (Dibattista et al., 2008) and in somatosensory neurons the elevation of cAMP increased the AP frequency after depolarizing steps due to an I_h-dependent increase in the membrane potential (Momin et al., 2008). Thus, cAMP-dependent changes in the gating of HCN channels were suggested to control the spontaneous activity of ORNs. The cAMP-dependent opening of I_h channels in ORNs of *M. sexta* might decrease the current threshold to evoke APs and thus would lead to an increase of the spontaneous AP frequency. Also in ORNs of moths I_h channels were identified. A HCN channel was cloned, electrophysiologically characterized and located in ORNs of *Heliothis virescens* (Krieger et al., 1999). For *M. sexta* cyclic nucleotide activated I_h currents were observed in patch

clamp recordings of cultured ORNs (Krannich, 2008). It remains to be studied whether I_h channels are indeed responsible for the setting of the resting potential and if they are involved in the control of the spontaneous activity in *M. sexta* ORNs.

Furthermore, 8bcAMP might affect the spontaneous activity via direct interaction with the putative OR83b analogue MsextaOR2 (Patch et al., 2009). Olfactory sensilla lacking OR83b receptors showed very little or no spontaneous AP activity (Larsson et al., 2004). In addition OR83b was activated after 8bcAMP application (Wicher et al., 2008) and the receptor complex consisting of OR47a and OR83b was shown to be spontaneously active (Sato et al., 2008). It is still unknown what physiological relevance the observed changes in the spontaneous AP frequency could have.

Time-dependency of 8bcAMP effects

Here, it was shown for the first time that cAMP is involved in the time-dependent modulation of insect odour transduction, possibly OA-dependently. Several earlier studies investigated the role of biogenic amines in the circadian regulation of the sensitivity of moths ORNs to pheromone. In *M. sexta* the level of OA in the hemolymph is regulated in a circadian rhythm with a maximum during the scotophase when the nocturnal moths showed maximal mating behaviour and with low levels during rest in the photophase (Lehman, 1990; Lingren et al., 1977). Also for *Trichoplusia ni* time-dependent changes in the OA levels in the hemolymph and brain were found which correlated with behavioural rhythms (Linn Jr et al., 1994; Linn Jr et al. 1996). In addition, OA perfusion increased the SP amplitude time-dependently with a maximum effect at ZT 8-11, but not at ZT 22-1 (Flecke and Stengl, 2009). OA application counteracted an endogenous desensitization occurring during the photophase, thus it was ineffective in the scotophase when the moths were maximally sensitive (Flecke and Stengl, 2009). In accordance with the time-dependency of the OA effects 8bcAMP increased the SP amplitude during the photophase but not during the scotophase and antagonized an endogenous shift to lower mean SP amplitudes in recordings with 10 µg BAL stimulation at ZT 1-4 and ZT 8-11. Furthermore, both OA and 8bcAMP antagonized an endogenous shift to lower mean spontaneous AP frequencies during the photophase. If the variations in the endogenous cAMP levels are correlated with the rhythms in OA concentrations, 8bcAMP could also be solely effective during low cAMP concentrations. However, there are also discrepancies between the time-dependency of OA and 8bcAMP effects. While the 8bcAMP-dependent increases with 1 µg BAL stimulation were almost equal in amplitude between ZT 1-4 and ZT 8-11, OA strongly increased the SP amplitude only at ZT 8-11, suggesting a time-dependent desensitization of OA receptors or adenylyl cylcases. In addition the 8bcAMP-dependent effect on the mean spontaneous AP frequency at ZT 8-11 was much weaker than the OA effect, suggesting an additional OA-dependent and time-dependent mechanism. Therefore, we currently investigate whether cAMP concentrations express a circadian rhythm and whether OA stimulates adenylyl cyclases time-dependently in the antenna of *M. sexta*.

In addition, our results add new evidence to the assumption that in moths circadian changes in the sensitivity to pheromone are also regulated at the periphery. In *D. melanogaster* circadian rhythms in EAGs are controlled by independent peripheral antennal pacemaker neurons (Tanoue et al., 2004). Furthermore these rhythms depended on G protein receptor kinase controlled accumulation of olfactory receptors in the dendrite of the sensilla (Tanoue et al., 2008). Also in moths circadian rhythms in EAGs and in the expression of olfactory receptors and different clock proteins in antenna were discovered (Merlin et al., 2007). For *M. sexta* PERIOD-like immunoreactivity was found in ORNs and their supporting cells (Schuckel et al., 2007), hinting that pheromone-sensitive ORNs are peripheral circadian pacemakers. This assumption is further supported by the finding of time-dependent differences in the strength and temporal resolution of BAL responses of single ORNs (Flecke and Stengl, 2009) and time-dependent endogenous changes in the SP amplitude, as shown here. Furthermore, we showed for the first time that ORNs adapt on the level of the spontaneous AP activity with beginning of the photophase. Interestingly, time-dependent changes in the spontaneous AP activity correlated with endogenous changes in the temporal resolution which were OA-, but not 8bcAMP-dependent. All these data suggest that also in *M. sexta* pheromone sensitivity and temporal resolution are controlled by a circadian clock at the periphery. Whether all ORNs in *M. sexta* are peripheral circadian pacemakers needs to be further investigated.

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References

Ache, B. W. and Young, J. M. (2005). Olfaction: diverse species, conserved principles. *Neuron* **48**, 417-430.

Baker, T. C. and Cardé, R. T. (1979). Endogenous and exogenous factors affecting periodicities of female calling and male sex pheromone response in *Grapholitha molesta* (Busck). *J. Insect Physiol.* **25**, 943-950.

Balfanz, S., Strünker, T., Frings, S. and Baumann A. (2005). A family of octopamine [corrected] receptors that specifically induce cyclic AMP production or Ca^{2+} release in *Drosophila*

melanogaster. *J. Neurochem.* **93**, 440-4451. Erratum in: *J. Neurochem.* **94**, 1168.

Bell, R. A. and Joachim, F. A. (1976). Techniques for rearing laboratory colonies of tobacco hornworms and pink bollworms. *Ann. Ent. Soc. Am.* **69**, 365-373.

Berridge, M. J. and Prince, W.T. (1972). Transepithelial potential changes during stimulation of isolated salivary glands with 5-hydroxytryptamine and cyclic AMP. *J. Exp. Biol.* **56**, 139-153.

Beyenbach, K. W. and Wieczorek, H. (2006). The V-type H^+ ATPase: molecular structure and function, physiological roles and regulation. *J. Exp. Biol.* **209**, 577-589.

Bischof, L. J. and Enan, E. E. (2004). Cloning, expression and functional analysis of an octopamine receptor from *Periplaneta americana*. *Insect Biochem. Mol. Biol.* **34**, 511-521.

Boekhoff, I., Michel, W. C., Breer, H. and Ache, B. W. (1994). Single odors differentially stimulate dual second messenger pathways in lobster olfactory receptor cells. *J. Neurosci.* **14**, 3304-3309.

Choi, M. Y., Tatsukia, S. and Boob, K. S. (1998). Regulation of sex pheromone biosynthesis in the oriental tobacco budworm, *Helicoverpa assulta* (Lepidoptera: Noctuidae). *J. Insect Physiol.* **44**, 653-658.

Dacks, A. M., Dacks, J. B., Christensen, T. A. and Nighorn, A. J. (2006). The cloning of one putative octopamine receptor and two putative serotonin receptors from the tobacco hawkmoth, *Manduca sexta*. *Insect Biochem. Mol. Biol.* **36**, 741-747.

Dames, P., Zimmermann, B., Schmidt, R., Rein, J., Voss, M., Schewe, B., Walz, B. and Baumann, O. (2006). cAMP regulates plasma membrane vacuolar-type H^+-ATPase assembly and activity in blowfly salivary glands. *Proc. Natl. Acad. Sci. USA* **103**, 3926-3931.

Dibattista, M., Mazzatenta, A., Grassi, F., Tirindelli, R. and Menini, A. (2008). Hyperpolarization-activated cyclic nucleotide-gated channels in mouse vomeronasal sensory neurons. *J. Neurophysiol.* **100**, 576-586.

Dolzer, J. (2002). Mechanisms of modulation and adaptation in pheromone-sensitive trichoid sensilla of the hawkmoth *Manduca sexta*. *Ph.D. Thesis* Philipps-Universität, Marburg, Germany http://archiv.ub.uni-marburg.de/diss/z2002/0185

Dolzer, J., Fischer, K. and Stengl, M. (2003). Adaptation in pheromone-sensitive trichoid sensilla of the hawkmoth *Manduca sexta*. *J. Exp. Biol.* **206**, 1575-1588.

Dolzer, J., Krannich, S. and Stengl, M. (2008). Pharmacological investigation of protein kinase C- and cGMP-dependent ion channels in cultured olfactory receptor neurons of the hawkmoth *Manduca sexta*. *Chem. Senses* **33**, 803-813.

Dolzer, J., Krannich, S., Fischer, K. and Stengl, M. (2001). Oscillations of the transepithelial potential of moth olfactory sensilla are influenced by octopamine and serotonin. *J. Exp. Biol.* **204**, 2781-2794.

Doolin, R. E. and Ache, B. W. (2005). Cyclic nucleotide signaling mediates an odorant-suppressible chloride conductance in lobster olfactory receptor neurons. *Chem. Senses* **30**, 127-135.

Evans, P. D. and Maqueira, B. (2005). Insect octopamine receptors: a new classification scheme based on studies of cloned *Drosophila* G-protein coupled receptors. *Invert. Neurosci.* **5**, 111-118.

Farooqui, T. (2007). Octopamine-mediated neuro-modulation of insect senses. *Neurochem. Res.* **32**, 1511-1529.

Flecke, C. and Stengl, M. (2009). Octopamine and tyramine modulate pheromone-sensitive olfactory sensilla of the hawkmoth *Manduca sexta* in a time-dependent manner. *J. Comp. Physiol. A*. DOI 10.1007/s00359-009-0429-4.

Flecke, C., Dolzer, J., Krannich, S. and Stengl, M. (2006). Perfusion with cGMP analogue adapts the action potential response of pheromone-sensitive sensilla trichoidea of the hawkmoth *Manduca sexta* in a daytime-dependent manner. *J. Exp. Biol.* **209**, 3898-3912.

Gisselmann, G., Marx, T., Bobkov, Y., Wetzel, C. H., Neuhaus, E. M., Ache, B. W. and Hatt, H. (2005). Molecular and functional characterization of an I(h)-channel from lobster olfactory receptor neurons. *Eur. J. Neurosci.* **21**, 1635-1647.

Gomez-Diaz, C., Martin, F. and Alcorta, E. (2004). The cAMP transduction cascade mediates olfactory reception in *Drosophila melanogaster*. *Behav. Genet.* **34**, 395-406.

Grohmann, L., Blenau, W., Erber, J., Erbert, P. R., Strünker, T., Baumann, A. (2003). Molecular and functional characterization of an octopamine receptor from honeybee (*Apis mellifera*) brain. *J. Neurochem.* **86**, 725-735.

Grosmaitre, X., Marion-Poll, F. and Renou, M. (2001). Biogenic amines modulate olfactory receptor neurons firing activity in *Mamestra brassicae*. *Chem. Senses* **26**, 653-661.

Han, K. A., Millar, N. S. and Davis, R. L. (1998). A novel octopamine receptor with preferential expression in *Drosophila* mushroom bodies. *J. Neurosci.* **18**, 3650-3658.

Hatt, H. and Ache, B. W. (1994). Cyclic nucleotide- and inositol phosphate-gated ion channels in lobster olfactory receptor neurons. *Proc. Natl. Acad. Sci. USA* **91**, 6264-6268.

Itagaki, H. and Conner, W. E. (1988). Calling behavior of *Manduca sexta* (L.) (Lepidoptera: Sphingidae) with notes on the morphology of the female sex pheromone gland. *Ann. Ent. Soc. Am.* **81**, 798-807.

Klein, U. (1992). The insect V-ATPase, a plasma membrane proton pump energizing secondary active transport: immunological evidence for the occurrence of a V-ATPase in insect ion-transporting epithelia. *J. Exp. Biol.* **172**, 345-354.

Klein, U. and Zimmermann, B. (1991). The vacuolar-type ATPase from insect plasma membrane: immunocytochemical localization in insect sensilla. *Cell Tissue Res.* **266**, 265-273.

Krannich, S. (2008). Electrophysiological and pharmacological characterization of ion channels involved in moth olfactory transduction cascades. *Ph.D. Thesis*, Philipps-University Marburg, http://archiv.ub.uni-marburg.de/diss/z2008/0727/

Krannich, S. and Stengl, M. (2008). Cyclic nucleotide-activated currents in cultured olfactory receptor neurons of the hawkmoth *Manduca sexta*. *J. Neurophysiol.* **100**, 2866-2877.

Krieger, J., Strobel, J., Vogl, A., Hanke, W. and Breer, H. (1999). Identification of a cyclic nucleotide- and voltage-activated ion channel from insect antennae. *Insect Biochem. Mol. Biol.* **29**, 255-267.

Larsson, M. C., Domingos, A. I., Jones, W. D., Chiappe, M. E., Amrein, H. and Vosshall, L. B. (2004). Or83b encodes a broadly expressed odorant receptor essential for *Drosophila* olfaction. *Neuron* **43**, 703-714.

Lehman, H. K. (1990). Circadian control of *Manduca sexta* flight. *Abstr. Soc. Neurosci.* **16**, 1334.

Lingren, P. D., Greene, G. L., Davis, D. R., Baumhover, A. H. and Henneberry, T. J. (1977). Nocturnal behavior of four lepidopteran pests that attack tobacco and other crops. *Ann. Ent. Soc. Am.* **70**, 161-167.

Linn, C. E. Jr. and Roelofs, W. L. (1986). Modulatory effects of octopamine and serotonin on male sensitivity and periodicity of response to sex pheromone in the cabbage looper moth, *Trichoplusia ni*. *Arch. Insect Biochem. Physiol.* **3**, 161-171.

Linn, C. E. Jr. and Roelofs, W. L. (1992). Role of photoperiod cues in regulating the modulatory action of octopamine on pheromone-response thresholds in the cabbage looper moth. *Arch. Insect Biochem. Physiol.* **20**, 285-302.

Linn, C. E. Jr., Campbell, M. G. and Roelofs, W. L. (1992). Photoperiod cues and the modulatory action of octopamine and 5-hydroxytryptamine on locomotor and pheromone response in male gypsy moths, *Lymantria dispar*. *Arch. Insect Biochem. Physiol.* **20**, 265-284.

Linn, C. E. Jr., Poole, K. R. and Roelofs, W. L. (1994). Studies on biogenic amines and their metabolites in nervous tissue and hemolymph of adult male cabbage looper moths I. Quantitation of photoperiod changes. *Comp. Biochem. Physiol.* **108**, 73-85.

Linn, C. E. Jr., Campbell, M. G., Poole, K. R., Wu, W.-Q. and Roelofs, W. L. (1996). Effects of photoperiod on the circadian timing of pheromone response in male *Trichoplusia ni*: Relationship to the modulatory action of octopamine. *J. Insect Physiol.* **42**, 881-891.

Martín, F., Charro, M. J. and Alcorta, E. (2001). Mutations affecting the cAMP transduction pathway modify olfaction in *Drosophila*. *J. Comp. Physiol. A.* **187**, 359-370.

Merlin, C., Lucas, P., Rochat, D., François, M. C., Maïbèche-Coisne, M. and Jacquin-Joly, E. (2007). An antennal circadian clock and circadian rhythms in peripheral pheromone reception in the moth *Spodoptera littoralis*. *J. Biol. Rhythms* **22**, 502-514.

Momin, A., Cadiou, H., Mason, A. and McNaughton, P. A. (2008). Role of the hyperpolarization-activated current I_h in somatosensory neurons. *J. Physiol.* **586**, 5911-5929.

Nakamura, T. (2000). Cellular and molecular constituents of olfactory sensation in vertebrates. *Comp. Biochem. Physiol. A* **126**, 17-32.

Ohtani, A., Arai, Y., Ozoe, F., Ohta, H., Narusuye, K., Huang, J., Enomoto, K., Kataoka, H., Hirota, A. and Ozoe, Y. (2006). Molecular cloning and heterologous expression of an alpha-adrenergic-like octopamine receptor from the silkworm *Bombyx mori*. *Insect Mol. Biol.* **15**, 763-772.

Patch, H. M., Velarde, R. A., Walden, K. K. and Robertson, H. M. (2009). A Candidate Pheromone Receptor and Two Odorant Receptors of the Hawkmoth *Manduca sexta*. *Chem. Senses* doi:10.1093/chemse/bjp002

Pittendrigh, C. S., Eichhorn, J. H., Minis, D. H. and Bruce, V. G. (1970). Circadian systems, VI. Photoperiodic time measurement in *Pectinophora gossypiella*. *Proc. Natl. Acad. Sci. USA*. **66**, 758-764.

Pophof, B. (2000). Octopamine modulates the sensitivity of silkmoth pheromone receptor neurons. *J. Comp. Physiol. A* **186**, 307-313.

Pophof, B. (2002). Octopamine enhances moth olfactory responses to pheromones, but not those to general odorants. *J. Comp. Physiol. A* **188**, 659-662.

Prince, W. T. and Berridge, M. J. (1972). The effects of 5-hydroxytryptamine and cyclic AMP on the potential profile across isolated salivary glands. *J. Exp. Biol.* **56**, 323-333.

Robinson, R. B. and Siegelbaum, S. A. (2003). Hyperpolarization-activated cation currents: from molecules to physiological function. *Annu. Rev. Physiol.* **65**, 453-480.

Rosén, W. (2002). Endogenous control of circadian rhythms of pheromone production in the turnip moth, *Agrotis segetum*. *Arch. Insect Biochem. Physiol.* **50**, 21-30.

Rosén, W. Q., Han, G. B. and Löfstedt, C. (2003). The circadian rhythm of the sex-pheromone-mediated behavioral response in the turnip moth, *Agrotis segetum*, is not controlled at the peripheral level. *J. Biol. Rhythms* **18**, 402-408.

Sasaki, M. and Riddiford, L. M. (1984). Regulation of reproductive behaviour and egg maturation in the tobacco hawk moth, *Manduca sexta*. *Physiol. Entomol.* **9**, 315-327.

Sato, K., Pellegrino, M., Nakagawa, T., Nakagawa, T., Vosshall, L. B. and Touhara, K. (2008). Insect olfactory receptors are heteromeric ligand-gated ion channels. *Nature* **452**, 1002-1006.

Schuckel, J., Siwicki, K. K. and Stengl, M. (2007). Putative circadian pacemaker cells in the antenna of the hawkmoth *Manduca sexta*. *Cell Tissue Res.* **330**, 271-278.

Smart, R., Kiely, A., Beale, M., Vargas, E., Carraher, C., Kralicek, A. V., Christie, D. L., Chen, C., Newcomb, R. D. and Warr, C. G. (2008). *Drosophila* odorant receptors are novel seven transmembrane domain proteins that can signal independently of heterotrimeric G proteins. *Insect Biochem. Mol. Biol.* **38**, 770-780.

Stengl, M., Ziegelberger, G., Boekhoff, I. and Krieger, J. (1999). Perireceptor events and transduction mechanisms in insect olfaction. In *Insect olfaction* (ed. B. S.Hansson BS), pp. 49-66. Berlin: Springer Verlag.

Tanoue, S., Krishnan, P., Chatterjee, A. and Hardin, P. E. (2008). G protein-coupled receptor kinase 2 is required for rhythmic olfactory responses in *Drosophila*. *Curr. Biol.* **18**, 787-794.

Tanoue, S., Krishnan, T., Krishnan, B., Dryer, S. E. and Hardin, P. E. (2004). Circadian clocks in antennal neurons are necessary and sufficient for olfaction rhythms in *Drosophila*. *Curr. Biol.* **14**, 638-649.

Thurm, U. (1972). The generation of receptor potentials in epithelial receptors. In *Olfaction and Taste IV* (ed. D. Schneider), pp. 95–101. Stuttgart: Wissenschaftliche Verlagsgesellschaft

Thurm, U. (1974). Mechanisms of electrical membrane responses in sensory receptors, illustrated by mechanoreceptors. In *Biochemistry of Sensory Functions* (ed. L. Jaenicke), pp. 367–390. Berlin: Springer Verlag

Villet, R. H. (1978). Mechanism of insect sex-pheromone sensory transduction: role of adenylate cyclase. *Comp. Biochem. Physiol.* **61**, 389-394.

Wicher, D., Schäfer, R., Bauernfeind, R., Stensmyr, M. C., Heller, R., Heinemann, S. H. and Hansson, B. S. (2008). *Drosophila* odorant receptors are both ligand-gated and cyclic-nucleotide-activated cation channels. *Nature* **452**, 1007-1011.

Wieczorek, H. (1992). The insect V-ATPase, a plasma membrane proton pump energizing secondary active transport: molecular analysis of electrogenic potassium transport in the tobacco hornworm midgut. *J. Exp. Biol.* **172**, 335-343.

Wieczorek, H., Putzenlechner, M., Zeiske, W. and Klein, U. (1991). A vacuolar-type proton pump energizes K^+/H^+ antiport in an animal plasma membrane. *J. Biol. Chem.* **266**, 15340-15347.

Wieczorek, H., Huss, M., Merzendorfer, H., Reineke, S., Vitavska, O. and Zeiske, W. (2003). The insect plasma membrane H^+ V-ATPase: intra-, inter-, and supramolecular aspects. *J. Bioenerg. Biomembr.* **35**, 359-366.

Curriculum vitae

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PERSONAL

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ACADEMIC APPOINTMENT

2003 –	Research Assistant, Philipps-Universität Marburg, Germany. Working as a Ph.D. student on time-dependent effects of cyclic nucleotides and biogenic amines on the pheromone transduction of the hawkmoth *Manduca sexta.*

DEGREE

2003	Diploma Title of the thesis: "Effects of cyclic nucleotides and tetraethylammonium on stimulated trichoid sensilla of the hawkmoth *Manduca sexta.*"

EDUCATION

2003 –	Ph.D. student: Philipps-Universität Marburg, Germany
1996 – 2002	Undergraduate: Philipps-Universität Marburg, Germany
1987 – 1996	High school: Melanchthon-Schule Steinatal, Willingshausen, Germany

AWARDS

2008	Stipend for the 28th Blankenese Conference – Sensory Signaling and Information Processing, Hamburg, Germany, € 700
2005	Stipend for the 25th Blankenese Conference – Signalling in Sensory Systems, Hamburg, Germany, € 500

SCIENTIFIC MEETINGS

2004	Organizing Committee, 15th Neurobiological Ph.D.-students Workshop, Marburg, Germany

INVITED PRESENTATIONS

2006	"Effects of cyclic nucleotides and octopamine on olfactory sensilla of the hawkmoth *Manduca sexta".* 99th DZG meeting Münster, Germany

TEACHING

Tutor in undergraduate courses:

Animal Physiology	Subject: Fundamentals of Neurophysiology Method: Extracellular recordings from earthworm giant fibers
Animal Physiology	Subject: Fundamentals of Sensory Physiology Method: ERG from locust eye
Neurophysiology	Subject: Sensory Physiology Method: Extracellular recordings from pheromone-sensitive trichoid sensilla of the hawkmoth *Manduca sexta*
Biology for medical students	Subject: Human sensory physiology Method: Behavioural experiments

PUBLICATIONS

Reviewed Articles:

Flecke C, Stengl M (2009) Perfusion with cAMP analogue affects pheromone-sensitive trichoid sensilla of the hawkmoth *Manduca sexta* in a time-dependent manner. Submitted to *Journal of Experimental Biology*

Flecke C, Stengl M (2009) Octopamine and tyramine modulate pheromone-sensitive olfactory sensilla of the hawkmoth *Manduca sexta* in a time-dependent manner. *J Comp Physiol A* DOI 10.1007/s00359-009-0429-4

Flecke C, Dolzer J, Krannich S, Stengl M (2006) Perfusion with cGMP analogue adapts the action potential response of pheromone-sensitive sensilla trichoidea of the hawkmoth *Manduca sexta* in a daytime-dependent manner. *J Exp Biol* 209:3898-3912

Abstracts:

Flecke C, Stengl M (2009) Daytime-dependent effects of cAMP and octopamine on the pheromone transduction of the hawkmoth *Manduca sexta*. 8th Meeting of the German Neuroscience Society, Göttingen, T19-11A

Flecke C, Stengl M (2008) Daytime-dependent modulation of the pheromone transduction in olfactory sensilla of the hawkmoth *Manduca sexta.* 28th Blankenese Conference "Sensory Signalling and Information Processing", Hamburg, Germany, p49

Flecke C, Stengl M (2007) Daytime-dependent modulation of the pheromone transduction in olfactory sensilla of the hawkmoth *Manduca sexta.* 7th Meeting of the German Neuroscience Society, Göttingen, T20-10B

Flecke C, Schuckel J, Siwicki Kathleen K, Stengl M (2005) The role of octopamine in the pheromone transduction of trichoid sensilla of *Manduca sexta.* 25th Blankenese Conference "Signalling in Sensory Systems", Hamburg, Germany, p31

Flecke C, Schuckel J, Seyfarth J, Stengl M (2005) The role of octopamine in the pheromone transduction of trichoid sensilla of *Manduca sexta*. 6th Meeting of the German Neuroscience Society Göttingen, Proc 6th German Neurosci Soc Conf, Thieme, Stuttgart, 130B, p533

Flecke C, Dolzer J, Stengl M (2004) Effects of cyclic nucleotides on cultured olfactory receptor neurons and on olfactory sensilla of the hawkmoth *Manduca sexta.* 7th International Congress of Neuroethology, Nyborg/Denmark, P113, po108

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Christian Flecke

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Erklärung

Ich versichere, dass ich meine Dissertation

Time-dependent modulation of pheromone-sensitive trichoid sensilla of the hawkmoth *Manduca sexta*
(Tageszeitabhängige Modulation von pheromonsensitiven Trichoidsensillen des Tabakschwärmers *Manduca sexta*)

selbstständig, ohne unerlaubte Hilfe angefertigt und mich dabei keiner anderen als der von mir ausdrücklich bezeichneten Quellen und Hilfen bedient habe.

Die Dissertation wurde in der jetzigen oder ähnlichen Form noch bei keiner anderen Hochschule eingereicht und hat noch keinen sonstigen Prüfungszwecken gedient.

Marburg, den 12. Mai 2009 _____

(Christian Flecke)